CERTIFICATE

This is to certify that ***Mr. DEVRAS S. MACHCHHAR*** *has prepared his thesis entitled "Evaluation Of Antihyperlipidemic Activity Of Various Combinations Of Safflower Oil And Rice Bran Oil On Triton WR– 1339 Induced Hyperlipidemia In Rats" in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under our guidance. He has carried out the work at the Department of Pharmacology, Institute of Pharmacy, Nirma University.*

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DECLARATION

I declare that the thesis entitled "Evaluation Of Antihyperlipidemic Activity Of Various Combinations Of Safflower Oil And Rice Bran Oil On Triton WR– 1339 Induced Hyperlipidemia In Rats" has been prepared by me under the guidance of Dr. Shital J. Panchal, Assistant Professor, and Ms. Shraddha V. Bhadada, Assistant Professor, Department of Pharmacology, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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LIST OF CONTENTS

LIST OF ABBREVIATIONS:-

Short form	Full form
ABCA1	ATP-binding cassette transporter A1
apo	Apolipoprotein
HDL	High-density lipoproteins
IDL	Intermediate-density lipoproteins
kDa	Kilodaltons
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoproteins
LPL	Lipoprotein lipase
VLDL	Very-low-density lipoproteins
Chol	Cholesterol
Lp(a)	Lipoprotein (a)
TG	Triglyceride
HL	Hepatic lipase
ACoA	Acetyl-coenzyme A
C	Cholesterol
CE	Cholesteryl ester
HMG-CoA reductase	3-hydroxy-3-methylglutaryl-coenzyme A reductase
MVA	Mevalonate

LIST OF TABLES

LIST OF FIGURES

1. ABSTRACT

Aim and objective: The present study was undertaken to evaluate the anti-hyperlipidemic activity of various combinations of Safflower oil (SO) and Rice bran oil (RBO) on triton WR 1339 induced hyperlipidemia in rats.

Materials and Methods: Wistar rats of either sex were utilized for the study. SO, RBO, Combinations of SO and RBO and atorvastatin were administered for 30 days. Hyperlipidemia was developed by single intraperitoneal injection of triton WR 1339 on 31st day. Rats were divided in 8 groups , Normal control (NC), triton WR 1339 control , triton WR 1339 + SO (7 ml/kg, Orally), triton WR 1339 + RBO (7 ml/kg, Orally) , triton WR 1339 + [SO+RBO] (2:8,7 ml/kg, Orally), triton WR 1339 + [SO+RBO] (3:7, 7ml/kg, Orally), triton WR 1339 + [SO+RBO] (5:5 ,7 ml/kg, Orally) and triton WR 1339 + atorvastatin (2 mg/kg, Orally). After 24 hours of triton injection, the blood was collected from the retro- orbital plexus of the rat for the estimation of lipid profile and animals were sacrificed. The liver was isolated and homogenated for estimation of oxidative stress parameters.

Results: The administration of triton WR -1339 showed significant increase in the level of the Total cholesterol (TC) , Triglyceride (TG) , Low- Density lipoprotein (LDL),Very low density lipoprotein (VLDL), Atherosclerotic index (A.I) and Malondialdehyde (MDA) and showed a significant decrease in the level of High Density lipoprotein (HDL) , Ratio of HDL/TC and Reduced glutathione (GSH)

Pretreatment with SO, RBO, their combinations and atorvastatin showed a significant reduction in the TC, TG, LDL, VLDL, A.I , MDA except SO which showed nonsignificant reduction in the TG and VLDL level as compared to triton WR 1339 control group. These all treatments also showed significant increase in the HDL, HDL / TC and GSH level except SO which showed nonsignificant increase in HDL, HDL/TC and GSH level as compared to triton WR 1339 control group.

Administration of combination of SO and RBO in 2:8, 3:7 and 5:5 proportion produced a significant decrease in the TC, TG and VLDL as compared to SO treated group. It produced a significant decrease in TC and LDL as compared to RBO treated

group. Administration of combination of SO and RBO in 2:8, 3:7, 5:5 proportion produced significant increase in HDL / TC as compared to SO treated group. Administration of combination of SO and RBO in 5:5 proportion produced a significant increase in GSH level as compared to SO treated group.

Conclusion : In the present study, maximum depression in Total cholesterol was seen in [SO + RBO] (5:5) proportion, maximum depression in Triglyceride and VLDL and maximum increase in HDL and HDL/TC was seen in [SO + RBO] (3:7) proportion, maximum depression in LDL and Atherosclerotic (A.I) was seen in [SO + RBO] (2:8) proportion. Maximum depression in Malondialdehyde (MDA) was seen in [SO + RBO] (2:8) proportion and maximum increase in Reduced glutathione (GSH) was seen in [SO + RBO] (5:5) proportion.

So, our study suggests that SO and RBO in 3:7 proportion is the optimum combination as it shows the maximum depression in TG and VLDL level and it shows the maximum increase in HDL and HDL/TC which may be beneficial in treatment of hyperlipidemia. However further study is required to confirm its mechanisms for the higher effect as compared to other combinations.

2. INTRODUCTION

The hyperlipidemia comprises a heterogeneous group of disorders whose characteristic expression is an elevation in the plasma concentration of cholesterol and/or triglyceride. **(Goldstein et al, 1973)**

Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis-associated conditions, such as coronary heart disease (CHD), ischemic cerebrovascular disease, and peripheral vascular disease. These conditions account for most morbidity and mortality among middle-aged and older adults. Hyperlipidemia (elevated levels of triglycerides or cholesterol) and reduced HDL-C levels occur as a consequence of several interrelated factors that affect the concentrations of the various plasma lipoproteins. These factors may be lifestyle or behavioral (*e.g.*, diet or exercise), genetic (*e.g.*, mutations in a gene regulating lipoprotein levels), or metabolic (*e.g.*, diabetes mellitus or other conditions that influence plasma lipoprotein metabolism). **(Brunton et al, 2008)**

Epidemiological studies have identified numerous risk factors for atheromatous disease. Some of these cannot be altered (e.g. a family history of ischaemic heart disease), but others are modifiable and are potential targets for therapeutic drugs. **(Rang et al, 2005).** The list of modifiable risk factors includes the raised low density lipoprotein, reduced high density lipoprotein, hypertension, diabetes mellitus, cigarette smoking, obesity, physical inactivity, raised C-reactive protein, raised homocystein.

The common risk factors for atherosclerosis increase production of reactive oxygen species (ROS) by endothelial, vascular smooth muscle, and adventitial cells. These ROS initiate processes involved in atherogenesis through several important enzyme systems, including xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and nitric oxide synthase **(Harrison et al, 2003)**

Recent studies have demonstrated that increased formation of free radicals/reactive oxygen species (ROS) contribute to cardiovascular disease (CVD) progression**.** **(Kaliora et al, 2006, Wattanapitayakul et al,2001)** .

Reactive oxygen species induce cardiac dysfunction & cardiac apoptosis and/or necrosis in heart failure**. (Griendling et al, 1998)** Reactive oxygen species are formed intracellularly and are controlled by antioxidant defense system.

The generation of large amount of reactive oxygen species can overwhelm the intracellular antioxidant defense, causing activation of lipid peroxidation, protein modification and DNA breaks**. (Hiroi et al, 1999)**.Reactive oxygen species induced depletion of antioxidants is a key factor for initiation of atherosclerosis and the development of CVD**. (kaliora et al,2006)** Disorders of lipid metabolism, hyperlipidemia, hypertension and obesity are associated with increased oxidative stress and overproduction of oxygen free radicals**. (Zalba et al, 2001)**.An excess of superoxide anions (O2 _) are further converted into other reactive oxygen species and among them hydroxyl radical (OH_) is more damaging to lipids and lipoproteins **(Rehman et al, 2003)**.Moreover, hyperlipidemia following oxidative stress may cause oxidative modifications in low density lipoproteins, which play an important role in the initiation and progression of atherosclerosis and related cardiovascular diseases. **(Parthasarthy et al,1992)**

Statins and fibric acid derivatives have complementary effects on mixed hyperlipidemia. However, such combination therapy increases the risk of myopathy, which may result in life-threatening rhabdomyolysis. Severe rhabdomyolysis with serious hepatocellular injury was observed one month after the combination antihyperlipidemic therapy was started. Assessment with the Naranjo probability scale determined that an adverse drug reaction was probable. The mechanism of this combined toxicity is difficult to clarify, although in vivo and in vitro studies to date have reported considerable data concerning antihyperlipidemic drug interactions. **(Hadim et al, 2007).** Resins are not absorbed so systemic toxicity is low but gastrointestinal symptoms-especially diarrhoea-are common and dose-related. High doses of Nicotinic acid can disturb liver function, impair glucose tolerance, and precipitate gout by increasing circulating urate concentration**.(Rang et al, 2005)**

The risk of Coronary artery disease (CAD) can be reduced by lowering serum cholesterol levels. **(Rossouw et al, 1990)**

It can be achieved by drug therapy and dietary manipulations. The use of dietary manipulation is less likely to cause undesirable consequences when compared with drug therapy. For instance, The American Heart Association guidelines for treating hypercholesterolemia have focused on dietary cholesterol & fat reduction.

The traditional cooking oil used in india include groundnut, coconut, sesame, mustard, safflower oil, while newer sources include rice bran oil, palmolein and soya bean oil. These oils are vegetable oils. **(Kuriyan et al, 2005)**

It has been reported that diabetic rats which were fed with sesame oil, when compared with controls (diabetic rats not receiving sesame oil), showed a significant reduction in the levels of blood Glucose. **(Ramesh et al ,2005)**

One of the most investigated properties of gamma- oryzanol present in rice bran oil is its anti-ulcerogenic property **(Mizuta et al.1978).** Fermented rice bran has a significant anti-stress and anti-fatigue effect**. (Kim et al, 2002)**

Dietary measures are initiated first unless the patient has evident coronary or peripheral vascular disease and may obviate the need for drugs. Patients with familial hypercholesterolemia or familial combined hyperlipidemia will always require drug therapy. Cholesterol, saturated fats and trans fats are the principal factors that increase LDL, whereas total fat, alcohol, and excess calories increase triglycerides. **(Katzung, 2007)**

In the previous studies about Safflower oil, it has been demonstrated that Supplementation of Safflower seed preparation like Safflower seed powder (5%, wt/wt; SSP), Safflower seed ethanol extract (0.15%, wt/wt; SSE), Safflower seed water extract (0.5%, wt/wt; SSW) for 5 weeks to the Sprague- Dawley rats showed the significant reduction in the plasma cholesterol concentration.

There were no significant differences in the concentrations of plasma HDL-C between the various groups. The hepatic triglyceride contents were significantly lower in both the SSE and SSW groups compared to control group. **(Moon et al, 2000)**

It has been demonstrated that administration of safflower oil diet for 4 month to the sprague-dawley rats showed significant reduction in the triglyceride level. **(Shimomura et al, 1990)**

It is reported that the administration of safflower oil diet for 7 days to the male wistar rats showed significant higher level of hepatic LDL receptor m-RNA expression which leads to the decrease in the LDL level & significant lower level of hepatic HMG- CoA reductase m-RNA expression which leads to decrease in the cholesterol level. **(Watanabe et al, 2000)**

It is reported that Kinobeon A which is isolated from cultured cells of safflower was shown to have significant inhibitory activity against lipid peroxidation in rat liver microsomes and shown to have effective O_2- scavenging activity. Anti-oxidative compounds isolated from safflower oil cake possess scavenging abilities against reactive oxygen species, such as O_2- and against α, α diphenyl- β- picrylhydrazyl (DPPH) radicals. **(Zhang et al, 1997)**

In the previous studies, it has been demonstrated that the rats fed with rice bran oil at 10% level for a period of eight weeks showed significant lower TC, LDL-C and VLDL-C plasma levels in groups kept on cholesterol-containing and cholesterol-free diets. HDL-C was increased, while TG showed a non-statistically significant decrease. **(Sharma et al, 1986)**

The use of rice bran oil for 3 months in patients with hyperlipidemia showed the significant reduction in the plasma total cholesterol and triglyceride level. The reduction in the plasma LDL cholesterol with rice bran oil was just short of statistical significance (p= 0.06). HDL cholesterol levels were unchanged. **(Kuriyan et al, 2005)**

It has been demonstrated that the administration of diet containing rice bran oil to the albino rats for 4 weeks showed the inhibitory activity on lipid peroxidation process & leads to increase in the Superoxide dismutase & peroxidase activity. **(Rana et al,2004).**

Safflower oil (SO) is belonging to the class of Semi-drying oils & it contains glycerides of palmitic (6.5%), stearic (3.0 %) , arachidic (0.296 %) , oleic (13.0 %) , linoleic (76-79 %) and linolenic acids (90.15 %). The polyunsaturated fatty acid content of the SO is highest (75%) and is said to responsible to control cholesterol level in blood and thereby reduce incidence of heart attacks **(Kokate et al, 2005).** PUFA lower TC and LDL-C. **(Mensink et al, 1992)**, it also increases the LDL apolipoprotein B (apoB) fractional catabolic rates (FCR). **(Shepherd et al, 1980)**

Rice bran oil (RBO) is belonging to the class of Non drying oil & it incorporates a rich unsaponifiable fraction mainly composed by phytosterols, 4 methyl- sterols tocotrienols, tocopherol and triterpene alcohols. It contains mainly oleic acid (38.4%), linoleic acid (34.4%) and α-linolenic acid (2.2%) as unsaturated fatty acids, and palmitic (21.5%) and stearic (2.9%) acids as saturated fatty acids **(Sayre et al, 1990).** Phytosterols, tocopherols and tocotrienols are supposed to contribute to antihyperlipidemic action of rice bran oil, while the particular fatty acid mono- and polyunsaturated composition seems not to be fundamental in its activity **(Rong et al, 1992).**

It is reported that blend of 7 parts of RBO with 3 parts of SO unexpectedly enhanced the cholesterol lowering potential of RBO in human study. The lowering effect of the blended oil was greater than that of the respective oils alone and other proportions of the blend were less effective in lowering serum cholesterol. The high linoleic acid content of SO in combination with the micronutrients of the RBO unsaponifiable fraction, acts synergistically to lower the serum cholesterol level. **(Suzuki et al, 1970)**

Presence of high amount of tocopherols and tocotrienols in RBO may improve the oxidative stability of the blends of SO and RBO. In addition to improving the plasma lipid profile, blending of RBO with SO can result in an economic advantage of lower prices. **(Sunitha et al, 1997)**

Reports showed the beneficial effects of combination of SO and RBO in various proportions like 2:8, 3:7, 5:5 but there is lack of animal experiments which give idea about the proportion of oil which shows the highest beneficial effect in hyperlipidemia.

Therefore, the present study was designed to evaluate and to optimize the anti- hyperlipidemic activity of various combinations of Safflower oil (SO) and Rice bran oil (RBO) on triton WR 1339 induced hyperlipidemic rats.

3. REVIEW OF LITERATURE

3.1 Epidemiology & Etiology of hyperlipidemia

3.1.1 Epidemiology of hyperlipidemia:

Hyperlipidemia has been ranked as one of the greatest risk factors contributing to prevalence and severity of coronary heart diseases **(Grundy, 1986)** Coronary heart disease, stroke, atherosclerosis and hyperlipidemia are the primary cause of death. **(Davey , 1993)**

Hyperlipidemia characterized by elevated serum total cholesterol and low density and very low density lipoprotein cholesterol and decrease high density lipoprotein are the risk factor for coronary heart diseases. Hyperlipidemia associated lipid disorders are considered to cause the atherosclerotic cardiovascular disease. **(Saravanan et al,2003)**

Hyperlipidemia characterized by hypercholesterolemia is the most prevalent indicator for susceptibility to cardiovascular disease. World health organization reports that high blood cholesterol contributes to approximately 56% cases of cardiovascular disease worldwide and causes about 4.4 million death each year. **(NCEP guidelines, 2002)**

Atherosclerosis, referred to as a "silent killer", is one of the leading causes of death in the developing countries like India. **(Ghatak et al, 1995)**

A relationship between total cholesterol and the development of coronary heart disease is recognized, as is its contribution to the 1 in 4 deaths from coronary heart disease that occurs each year in the UK. A reduction in the mean level of total cholesterol in the population will reduce the development of coronary atherosclerosis and the prevalence of coronary heart disease. **(Walker et al, 2007)**

Coronary heart disease continues to be the number one cause of death in most Northern European, North American and other industrialized Caucasian societies. By the age of 60, every fifth man and one in 17 women have some form of this disease. **(Castelli et al, 1984)**

3.1.2 Etiology of hyperlipidemia

It can be caused by primary causes or secondary causes.

1. **Primary causes:**

   (i) these can be familial or genetic due to single gene defect, OR **(ii)** can be multifactorial or polygenic which have multiple genetic ,dietary and physical activity related causes.

2. **Secondary causes :**

   These hyperlipoproteinaemias are associated with some disease, e.g., diabetes, myxoedema, nephritic syndrome, chronic alcoholism, or drugs like oral contraceptives and β- blockers etc**. (Sharma et al, 2007)**

3.1.3 Modifiable risk factors for atheromatous disease: (Rang et al, 2005)

- Raised low-density lipoprotein cholesterol
- Reduced high-density lipoprotein cholesterol
- Hypertension
- Diabetes mellitus
- Cigarette smoking
- Obesity
- Physical inactivity
- Raised C-reactive protein
- Raised homocystein

3.2 Pathophysiology:

Lipoprotein metabolism:

3.2.1 Lipoprotein classification and composition: (Rang et al,2005, Katzung , 2007)

Lipids and cholesterol are transported through the blood stream as macromolecular complexes of lipid and protein known as lipoproteins.These consist of a central core of hydrophobic lipid(including triglycerides and cholesteryl esters) encased in a hydrophilic coat of polar phospholipid, free cholesterol and apolipoprotein.

There are four main classes of lipoprotein, differing in the relative proportion of the core lipids and in the type of apoprotein. They also differ in size and density, and this letter property,as measured by ultracentrifugation ,is the basis for classification into :-

- Chylomicrons.
- Very low- density lipoprotein.
- LDL-C particles.
- HDL-C particles.

A. Chylomicrons

They are formed in the intestine and carry triglycerides of dietary origin, unesterified cholesterol, and cholesteryl esters. They transit the thoracic duct to the blood stream.
Triglycerides are removed in extrahepatic tissues through a pathway shared with VLDL that involves hydrolysis by the lipoprotein lipase system. Decrease in particle diameter occurs as triglycerides are depleted.
Surface lipids and small lipoprotein are transferred to HDL. The resultant chylomicron remnants are taken up by receptor mediated endocytosis into hepatocytes.

B. Very low density lipoprotein

They are secreted by liver and export triglycerides to peripheral tissues. VLDL triglycerides are hydrolyzed by LPL-yielding free fatty acids for storage in adipose tissue and for oxidation in tissues such as cardiac and skeletal muscles.

Depletion of triglycerides produces remnants (IDL), some of which undergo endocytosis directly by liver. The remainder is converted to LDL by further removal of triglycerides mediated by hepatic lipase.

This process explains the "beta shift " phenomenon, the increase of LDL (beta lipoprotein) in serum as hypertriglyceridemia. Increased level of LDL can also result from increased secretion of VLDL and from decreased LDL catabolism.

C. LDL – C Particles

LDL is catabolized chiefly in hepatocytes and other cells by receptor-mediated endocytosis. Cholesteryl esters from LDL are hydrolyzed, yielding free cholesterol for the synthesis of cell membranes. Cells also obtain cholesterol by synthesis via a pathway involving the formation of mevalonic acid by HMG-CoA reductase.

Production of this enzyme and of LDL receptors is transcriptionally regulated by the content of cholesterol in the cell. Normally, about 70% of LDL is removed from plasma by hepatocytes. Even more cholesterol is delivered to the liver via IDL and chylomicrons. Unlike other cells, hepatocytes can eliminate cholesterol by secretion in bile and by conversion to bile acids.

D.HDL-C Particles

The apoproteins of HDL are secreted by the liver and intestine. Much of the lipid comes from the surface monolayers of chylomicrons and VLDL during lipolysis. HDL also acquires cholesterol from peripheral tissues, protecting the cholesterol homeostasis of cells. Free cholesterol is transported from the cell membrane by a transporter, ABCA1, acquired by a small particle termed prebeta-1 HDL, and then esterified by lecithin: cholesterol acyltransferase (LCAT), leading to the formation of larger HDL species.

Cholesterol is also exported from macrophages by the ABCG1 transporter to large HDL particles. The cholesteryl esters are transferred to VLDL, IDL, LDL, and chylomicron remnants with the aid of cholesteryl ester transfer protein (CETP). Much of the cholesteryl ester thus transferred is ultimately delivered to the liver by endocytosis of the acceptor lipoproteins. HDL can also deliver cholesteryl esters

directly to the liver via a docking receptor (scavenger receptor, SR-BI) that does not cause endocytosis of the lipoproteins.

***Table 1*: Characteristics of Plasma Lipoproteins : (Brunton et al,2008)**

Lipoprotein Class	Density of Flotation, g/mL	Major Lipid Constituent	TG:Chol Ratio	Significant Apoproteins	Site of Synthesis	Mechanism(s) of Catabolism
Chylomicrons and remnants	<<1.006	Dietary triglycerides and cholesterol	10:1	B-48, E, A-I, A-IV, C-I, C-II, C-III	Intestine	Triglyceride hydrolysis by LPL ApoE-mediated remnant uptake by liver
VLDL	<1.006	"Endogenous" or hepatic triglycerides	5:1	B-100, E, C-I, C-II, C-III	Liver	Triglyceride hydrolysis by LPL
IDL	1.006–1.019	Cholesteryl esters and "endogenous" triglycerides	1:1	B-100, E, C-II, C-III	Product of VLDL catabolism	50% converted to LDL mediated by HL, 50% apoE-mediated uptake by liver 50% apoE-mediated uptake by liver
LDL	1.019–1.063	Cholesteryl esters	NS	B-100	Product of VLDL catabolism	ApoB-100–mediated uptake by LDL receptor (~75% in liver)
HDL	1.063–1.21	Phospholipids, cholesteryl esters	NS	A-I, A-II, E, C-I, C-II, C-III	Intestine, liver, plasma	Complex: Transfer of cholesteryl ester to VLDL and LDL Uptake of HDL cholesterol by hepatocytes
Lp(a)	1.05–1.09	Cholesteryl esters	NS	B-100, apo(a)	Liver	Unknown

Lipoprotein families and its clinical significance :

Classification of plasma lipoproteins on the basis of apolipoprotein (apo) composition recognizes two lipoprotein (Lp) classes, one of which is characterized by apoA-I and the other by apoB as major protein constituents.

The former lipoprotein class consists of three major subclasses referred to (according to their apolipoprotein constituents) as Lp-A-I, Lp-A-I:A-II, and Lp-A-II, and the latter one of five subclasses called Lp-B, Lp-B:E, Lp-B:C, Lp-B:C:E, and Lp-A-II:B:C:D:E.

As polydisperse systems of particles, the apoA-I-containing lipoproteins overlap in high-density segments and apoB- containing lipoproteins in low-density segments of the density gradient. Each subclass is characterized by a specific chemical composition and metabolic property.

Normolipidemia and dyslipoproteinemias are characterized by quantitative rather than qualitative differences in the levels of apoA- and apoB-containing subclasses.

Furthermore, apoA-containing subclasses seem to differ with respect to their relative antiatherogenic capacities, and apoB-containing subclasses regarding their relative atherogenic potentials.

Whereas Lp-A-I may have a greater antiatherogenic capacity than other apoA-containing subclasses, the cholesterol-enriched Lp-B:C appears to be the most atherogenic subclass among apoB-containing lipoprotein families.

The use of pharmacologic and/or dietary interventions to treat dyslipoproteinemias has already shown that these therapeutic modalities may affect selectively individual apolipoprotein-defined lipoproteins, and thus allow the selection of individualized treatments targeted at decreasing harmful and/or increasing beneficial lipoprotein subclasses**. (Alaupovic et al, 2003)**

***Table 2* : Apolipoproteins : (Brunton et al, 2008)**

Apolipoprotein	Average Concentration, mg/dL	Chromosome	Molecular Mass, kDa	Sites of Synthesis	Functions
ApoA-I	130	11	~29	Liver, intestine	Structural in HDL; LCAT cofactor; ligand of ABCA1 receptor; reverse cholesterol transport
ApoA-II	40	1	~17	Liver	Forms –*S*–*S*– complex with apoE-2 and E-3, which inhibits E-2 and E-3 binding to lipoprotein receptors
ApoA-V	<1	11	~40	Liver	Modulates triglyceride incorporation into hepatic VLDL; activates LPL
ApoB-100	85	2	~513	Liver	Structural protein of VLDL, IDL, LDL; LDL receptor ligand
ApoB-48	Fluctuates according to dietary fat intake	2	~241	Intestine	Structural protein of chylomicrons
ApoC-I	6	19	~6.6	Liver	LCAT activator; modulates receptor binding of remnants
ApoC-II	3	19	8.9	Liver	Lipoprotein lipase cofactor
ApoC-III	12	11	8.8	Liver	Modulates receptor binding of remnants
ApoE	5	19	34	Liver, brain, skin, gonads, spleen	Ligand for LDL receptor and receptors binding remnants; reverse cholesterol transport (HDL with apoE)
Apo(a)	Variable (under genetic control)	6	Variable	Liver	Modulator of fibrinolysis

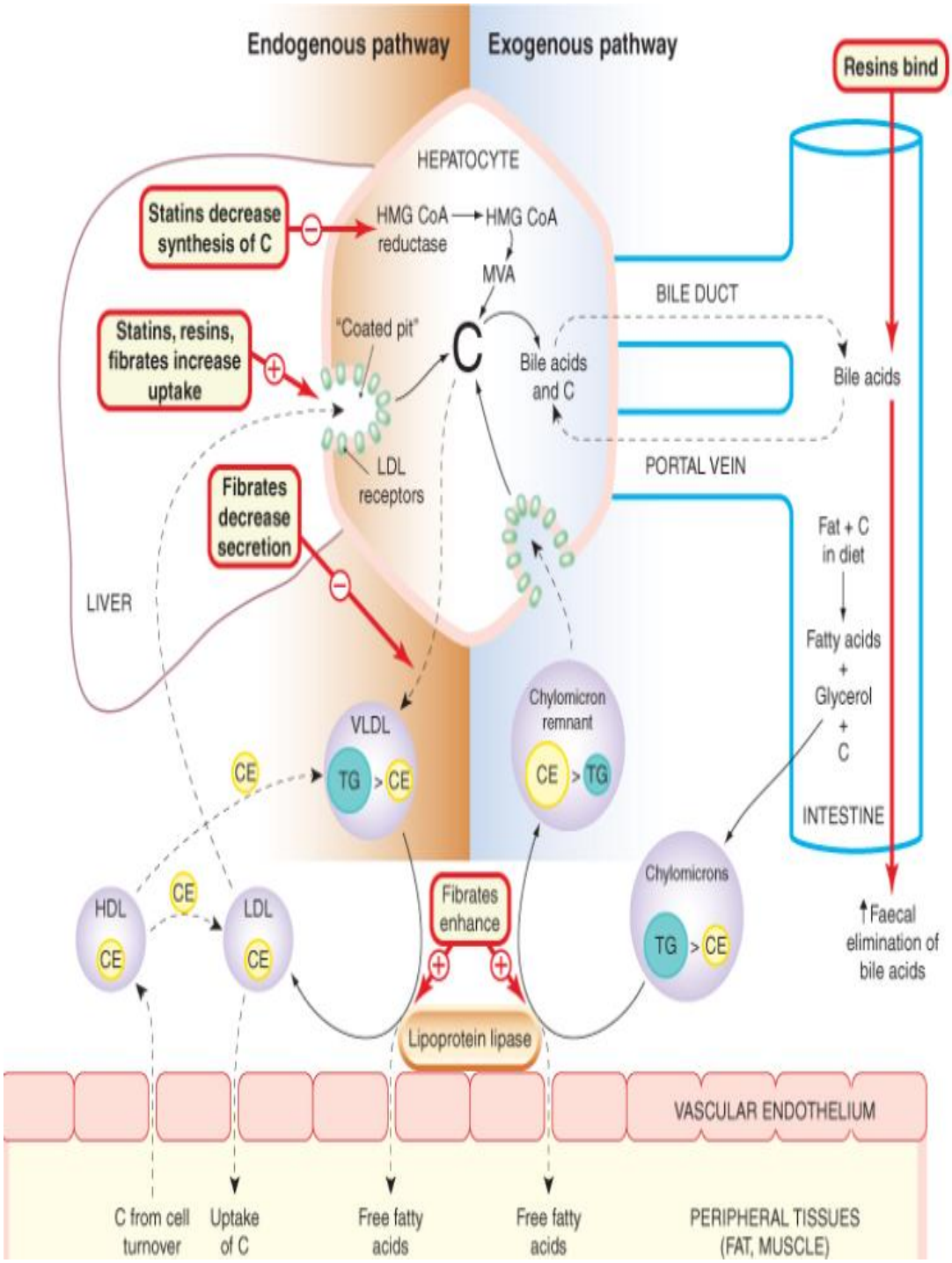

***Figure 1*: Schematic diagram of cholesterol transport in the tissues, with sites of action of the main drugs affecting lipoprotein metabolism.**

Each class of lipoprotein has a specific role in lipid transport, and there are different pathways for exogenous and for endogenous lipids, as well as a pathway for reverse cholesterol transport. The pathways are distinguished by the main apoproteins (apoB-48, apoB-100 and apoA1, respectively) that are ligands for the key receptors.

3.2.2 Transport of dietary lipids (Exogenous pathway) :

Cholesterol and triglycerides absorbed from the ileum are transported as chylomicrons (diameter 100-1000 nm), in lymph and then blood, to capillaries in muscle and adipose tissue. Here, triglycerides are hydrolysed by lipoprotein lipase, and the tissues take up the resulting free fatty acids and glycerol.

The chylomicron remnants (diameter 30-50 nm),still containing their full complement of cholesteryl esters, pass to the liver, bind to receptors on hepatocytes and undergo endocytosis. Cholesterol liberated in hepatocytes is stored, oxidised to bile acids, secreted unaltered in bile, or can enter the endogenous pathway.

3.2.3 Transport of dietary lipids (Endogenous pathway) :

In the endogenous pathway, cholesterol and newly synthesised triglycerides are transported from the liver as VLDL (diameter 30-80 nm) to muscle and adipose tissue, where triglyceride is hydrolysed to fatty acids and glycerol; these enter the tissues as described above. During this process, the lipoprotein particles become smaller (diameter 20-30 nm) but retain a full complement of cholesteryl esters.

Consequently, they increase in density to intermediate-density cholesterol and ultimately LDL-C particles. LDL-C provides the source of cholesterol for incorporation into cell membranes and for synthesis of steroids but is also key in atherogenesis, as described above. Cells take up LDL-C by endocytosis via LDL receptors that recognise LDL apolipoproteins.

Some drugs (notably statins) reduce circulating LDL-C by inhibiting endogenous cholesterol synthesis and stimulating the synthesis of hepatic LDL receptors. Cholesterol can return to plasma from the tissues in HDL particles (diameter 7-20 nm).

Cholesterol is esterified with long-chain fatty acids in HDL particles, and the resulting cholesteryl esters are transferred to VLDL or LDL particles by a transfer protein

present in the plasma and known as cholesteryl ester transfer protein (CETP). Lipoprotein(a), or Lp(a), is a species of LDL that is strongly associated with atherosclerosis and is localised in atherosclerotic lesions.Lp(a) contains a unique apoprotein, apo(a), with structural similarities to plasminogen . Lp(a) competes with and inhibits the binding of plasminogen to its receptors on the endothelial cell. Plasminogen is normally the substrate for plasminogen activator, which is secreted by and bound to endothelial cells, generating the fibrinolytic enzyme plasmin.The effect of the binding of Lp(a) is that less plasmin is generated, fibrinolysis is inhibited and thrombosis promoted. **(Rang et al, 2005)**

3.2.4 HDL metabolism and Reverse cholesterol transport :

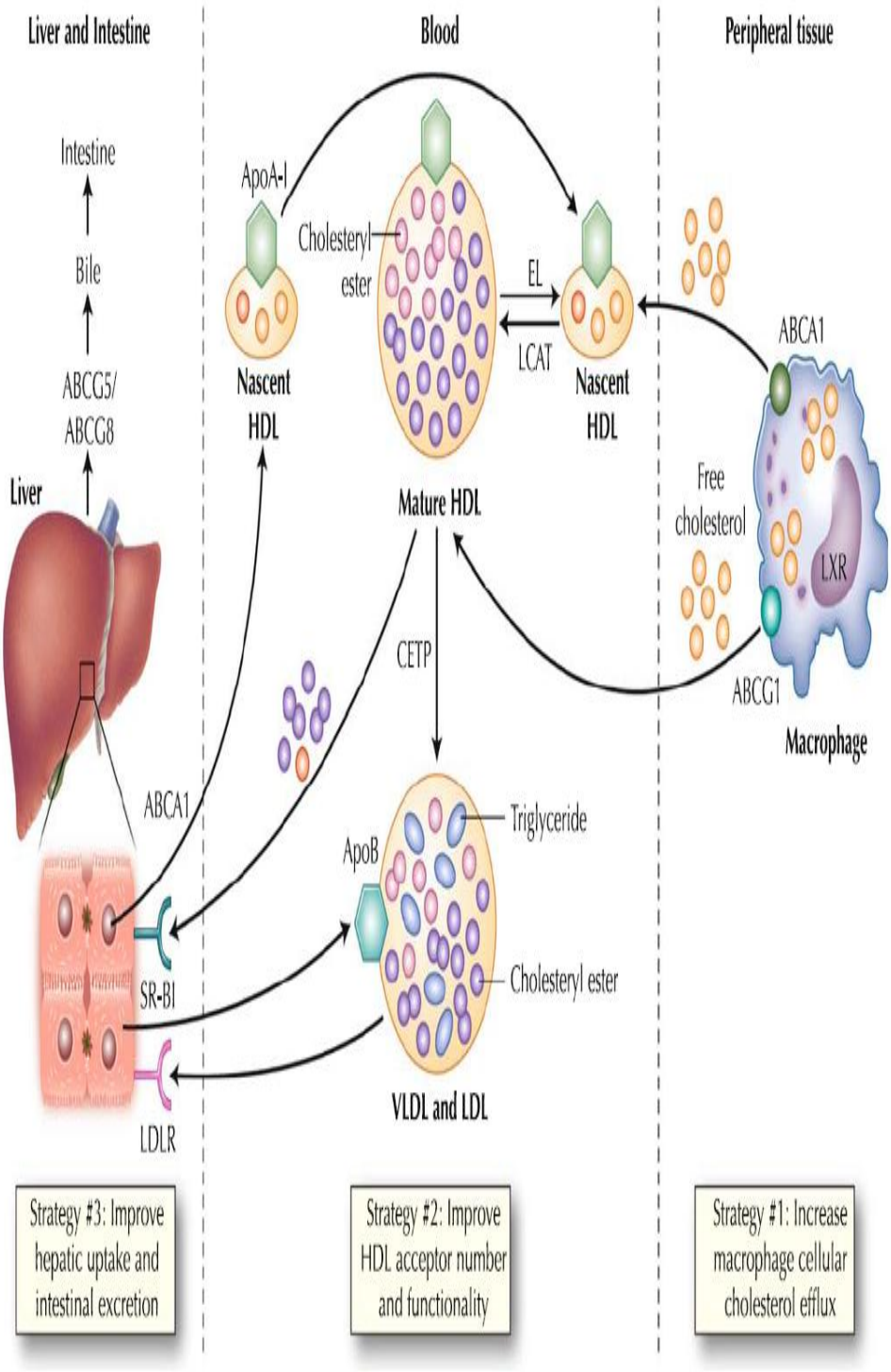

Figure 2: **Physiology of macrophage reverse cholesterol transport**

Reverse cholesterol transport is a multi-step process resulting in the net movement of cholesterol from peripheral tissues back to the liver via the plasma compartment.

Cellular cholesterol efflux is mediated by HDL, acting in conjunction with the cholesterol esterifying enzyme, lecithin: cholesterol acyltransferase. Cholesteryl ester accumulating in HDL can then follow a number of different fates: uptake in the liver in HDL containing apolipoprotein (particle uptake) by LDL receptors, selective uptake of HDL cholesteryl ester in liver or other tissues involving scavenger receptor B1, or transfer to triglyceride-rich lipoproteins as a result of the activity of cholesteryl ester transfer protein, with subsequent uptake of triglyceride-rich lipoprotein remnants in the liver.

Over-expression of the major HDL apoprotein, apolipoprotein A-I, is clearly anti-atherogenic. However, over- or under-expression of molecules such as cholesteryl ester transfer protein, which have opposite effects on HDL levels and reverse cholesterol transport, suggest that both HDL levels as well as the dynamics of cholesterol movement through HDL are involved in the anti-atherogenic actions of HDL. **(Tall ,1998)**

3.3 Classification of Hyperlipidemia :

Hyperlipidemias are classified according to the **Fredrickson classification** which is based on the pattern of lipoproteins on electrophoresis or ultracentrifugation. It was later adopted by the World Health Organization (WHO). It does not directly account for HDL, and it does not distinguish among the different genes that may be partially responsible for some of these conditions. It remains a popular system of classification, but is considered dated by many.

Fredrickson classification of Hyperlipidemias : **(Frederickson et al , 1965)**

Hyperlipoproteinemia type I :

This very rare form (also known as Buerger-Gruetz syndrome, primary hyperlipoproteinaemia, or familial hyperchylomicronemia) is due to a deficiency of lipoprotein lipase (LPL) or altered apolipoprotein C2, resulting in elevated chylomicrons, the particles that transfer fatty acids from the digestive tract to the liver. Its prevalence is 0.1% of the population.

Hyperlipoproteinemia type II :

Hyperlipoproteinemia type II, by far the most common form, is further classified into type IIa and type IIb, depending mainly on whether there is elevation in the triglyceride level in addition to LDL cholesterol.

Type IIa :

This may be sporadic (due to dietary factors), polygenic, or truly familial as a result of a mutation either in the LDL receptor gene on chromosome 19 (0.2% of the population) or the ApoB gene (0.2%). The familial form is characterized by tendon xanthoma, xanthelasma and premature cardiovascular disease.

Type IIb :

The high VLDL levels are due to overproduction of substrates, including triglycerides, acetyl CoA, and an increase in B-100 synthesis. They may also be caused by the decreased clearance of LDL. Prevalence in the population is 10%

Treatment:

While dietary modification is the initial approach, many patients require treatment with statins (HMG-CoA reductase inhibitors) to reduce cardiovascular risk. If the triglyceride level is markedly raised, fibrates may be preferable due to their beneficial effects.

Combination treatment of statins and fibrates, while highly effective, causes a markedly increased risk of myopathy and rhabdomyolysis and is therefore only done under close supervision. Other agents commonly added to statins are ezetimibe, niacin and bile acid sequestrants. There is some evidence for benefit of plant sterol-containing products and ω_3-fatty acids . **(Thompson, 2004)**

Hyperlipoproteinemia type III :

This form is due to high chylomicrons and IDL (intermediate density lipoprotein).

Also known as broad beta disease or dysbetalipoproteinemia, the most common cause for this form is the presence of ApoE E2/E2 genotype. It is due to cholesterol-rich VLDL (β-VLDL). Prevalence is 0.02% of the population.

Hyperlipoproteinemia type IV :

This form is due to high triglycerides. It is also known as hypertriglyceridemia (or pure hypertriglyceridemia). According to the NCEP-ATPIII definition of high triglycerides (>200 mg/dl), prevalence is about 16% of adult population.

Hyperlipoproteinemia type V :

This type is very similar to type I, but with high VLDL in addition to chylomicrons.

Unclassified forms :

Non-classified forms are extremely rare:

- Hypo-alpha lipoproteinemia
- Hypo-beta lipoproteinemia (prevalence 0.01-0.1%)

3.4 Treatment of hyperlipidemia:

Table 3 : National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATPIII) guidelines for desirable serum lipid levels in adults : (NCEP guidelines, 2002)

LDL CHOLESTEROL	
<100 mg/dl	Optimal
100-129 mg/dl	Near optimal /Above optimal
130-159 mg/dl	Borderline high
160-189 mg/dl	High
≥190 mg/dl	Very high
TOTAL CHOLESTEROL	
<200 mg/dl	Desirable
200-239 mg/dl	Borderline high
≥240 mg/dl	High
HDL CHOLESTEROL	
<40 mg/dl	Low
≥60 mg/dl	High
TRIGLYCERIDE LEVELS	
<150 mg/dl	Normal
150-199 mg/dl	Borderline high
200-499 mg/dl	High

3.4.1 Non- Pharmacological Treatment :

Dietary management in hyperlipoproteinemia :

1. Restriction of fat calories to less than 20 % of total calories.

2. Saturated, Monounsaturated and polyunsaturated fats should form 1/3rd each of the total dietary fat.

3. Increased consumption of vegetables, fresh fruit, cereals, nuts especially walnuts and almonds (in moderation) and whole grain products.

4. Increased consumption of fish, where possible/permitted.

5. Drastic reduction in alcohol consumption.**(Satoskar et al, 2009)**

Exercise :

Moderate amounts of aerobic exercise (brisk walking, jogging, swimming, cycling) on a regular basis have a desirable effect on the lipid profile of an individual. These beneficial effects have been demonstrated within 2 months in middle aged men exercising for 30 minutes, three times a week. Current advice for adults who are not routinely active is to undertake 30 minutes of moderate intensity activity on at least 5 days of the week.

For active individuals, additional aerobic exercise of vigorous intensity is recommended for 20-30 minutes three times a week. Exercise per se probably has little effect on total cholesterol levels in the absence of a reduction in body weight, body fat or dietary fat. Perhaps the most important effect of regular exercise is to raise levels of HDL-C in a dose dependent manner according to energy expenditure**. (Durstine et al, 2002)**

Stanol esters & plant sterols :

The availability of margarines and other foods enriched with plant sterols or stenol esters increases the likelihood that LDL-C can be reduced by dietary change. Both Stenol esters and Plant sterols at a maximum effective dose of 2g/day inhibit cholesterol absorption from the gastrointestinal tract and reduced LDL-C by an average of 10 %.

They compete with cholesterol for incorporation into mixed micelles, thereby impairing its absorption from the intestine. However, as with other dietary changes the reduction seen varies between individuals and is probably dependent on the initial cholesterol level. Single meal studies show that phyto-sterols are bioactive at doses as low as 150 mg and the small amounts that naturally occur in foods are also probably important in the management of total cholesterol.

Anti-oxidants :

Antioxidants occur naturally in fruit and vegetables and are important components of a healthy diet. Their consumption is thought to be beneficial in reducing the formation of atherogenic, oxidized LDL-C.

Primary and secondary prevention trials with anti-oxidant vitamin supplements, however, have not been encouraging. Neither vitamin E nor β carotene supplements would appear to reduce the risk of coronary heart disease but likewise have not been shown to be harmful. **(Walker et al,2007)**

3.4.2 Pharmacological Treatment:

The recent guidelines for detection and treatment of hypercholesterolemia together with specific therapeutic goals have stimulated interest in, and use of, lipid lowering agents. The last decade has seen an explosive growth in the drug discovery area which is now translating into clinical trials with many new lipid lowering agents.

In addition, clinical trials assessing clinical outcomes and cost effectiveness are resulting in a changing approach to how current lipid lowering drugs are used, especially in terms of dosing and combination therapies.

At present only four classes of lipid altering agents remain in wide use; bile acid binding resins, niacin, fibrates and HMG CoA reductase inhibitors. Although only a decade has passed since the first HMG CoA reductase inhibitor, lovastatin, entered clinical trials this group of compounds are not only the most widely used lipid lowering agents, but more than six such agents have been or are currently being developed.

In addition to pharmacological therapies, there have been significant advances in the non-pharmacological treatment of hypercholesterolemia, the most important being LDL-receptor gene replacement and selective LDL-apheresis**. (Stein et al, 1994)**

3.4.2.1 Current srugs therapy used to treat hyperlipidemia:

HMG-COA REDUCTASE INHIBITORS : STATINS

Statins, inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, have revolutionized the treatment of hypercholesterolemia. They are the most efficient agents for reducing plasma cholesterol, being also appreciated for their good tolerance.

Angiographic studies have demonstrated that these compounds reduce the progression and may induce the regression of atherosclerosis. These effects were translated in significant cardiovascular morbidity and mortality reductions in many clinical trials**. (Vaughan et al, 2000)**

Recently completed primary and secondary intervention trials have shown that the significant reductions in low-density lipoprotein (LDL) cholesterol achieved with statins result in significant reductions in morbidity and mortality associated with coronary artery disease as well as reductions in the incidence of stroke and total mortality.

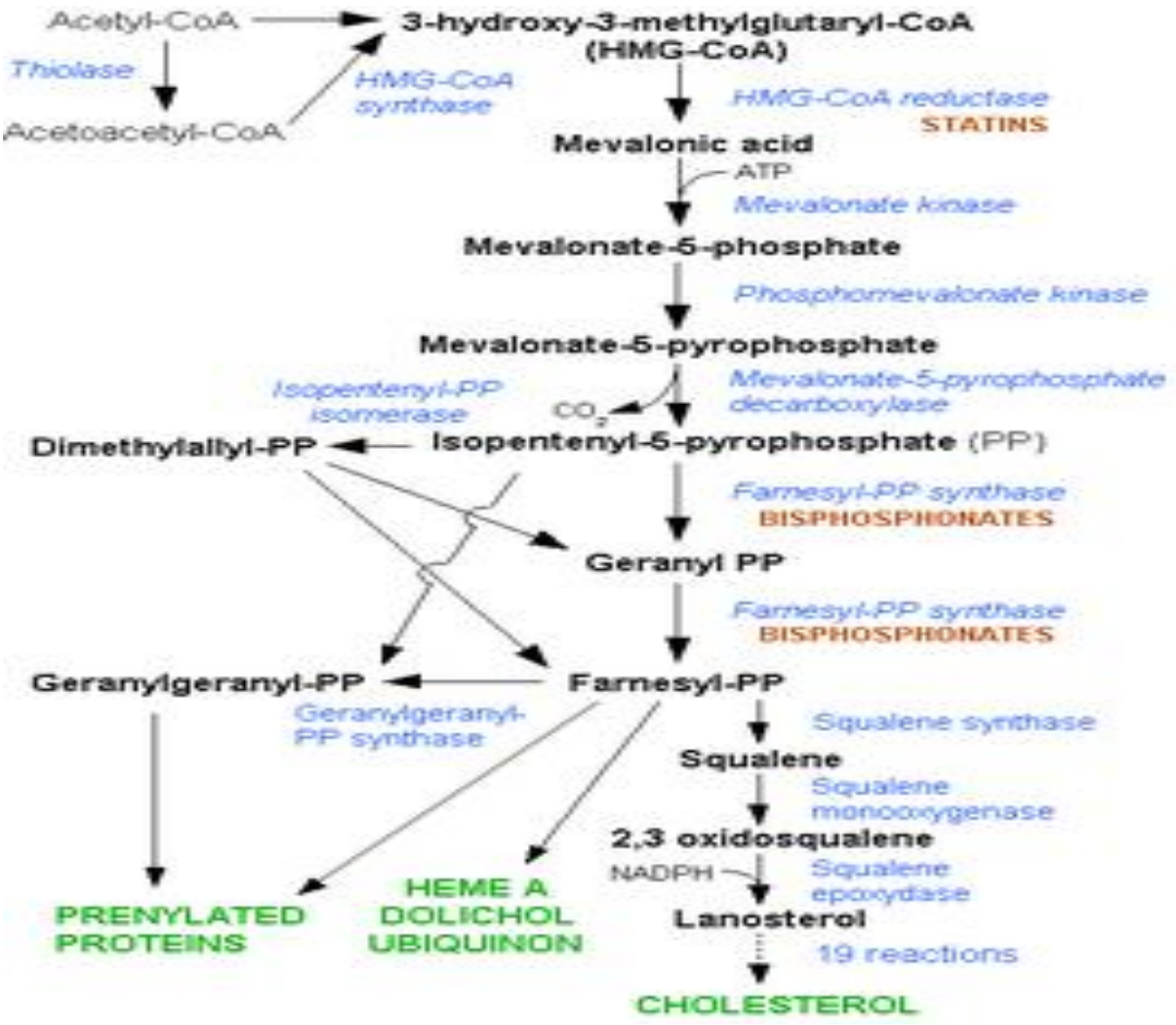

Figure 3 : **Pathway of Cholesterol biosynthesis**

The beneficial effects of the HMGCoA reductase inhibitors are usually attributed to their capacity to reduce the endogenous cholesterol synthesis, by competingly inhibiting the principal enzyme involved. **(Hunninghake , 1992)**

Since mevalonate, the product of HMG CoA reductase reaction, is the precursor not only for cholesterol, but also for many other nonsteroidal isoprenoidic compounds, inhibition of this key enzyme may result in pleiotropic effects.

They have been divided into two categories, involving: directly lipids, or intracellular signaling pathways. The first category includes: inhibition of cholesterol biosynthesis, increased uptake and degradation of low density lipoproteins (LDL), inhibition of the secretion of lipoproteins, inhibition of LDL oxidation, and inhibition of the scavenger receptors expression. **(Bellosta et al,2000)**

Inhibition of HMG CoA reductase :

Statins target hepatocytes and inhibit HMG-CoA reductase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. The statins do more than just compete with the normal substrate in the enzymes active site. They alter the conformation of the enzyme when they bind to its active site.

This prevents HMG-CoA reductase from attaining a functional structure. The change in conformation at the active site makes these drugs very effective and specific. Binding of statins to HMGCoA reductase is reversible, and their affinity for the enzyme is in the nanomolar range, as compared to the natural substrate, which has micromolar affinity. **(Corsini et al,1999)**

In patients at high risk of coronary artery disease but without evidence of atherosclerosis, treatment is designed to prevent the premature development of coronary artery disease, whereas in those with hypertriglyceridemia, treatment aims to prevent the development of hepatomegaly, splenomegaly, and pancreatitis.

Particular emphasis is given to cerivastatin, a new HMG-CoA reductase inhibitor that combines potent cholesterol-lowering properties with significant triglyceride-reducing effects.

Such benefits occur early in the course of statin therapy and have led to suggestions that these drugs may possess antiatherogenic effects over and above their capacity to lower atherogenic lipids and lipoproteins.

Experimental studies have also shown statin-induced improvements in endothelial function, decreased platelet thrombus formation, improvements in fibrinolytic activity, and reductions in the frequency of transient myocardial ischemia**.** **(Farnier et al,1998).**

Statins modulate a series of processes leading to reduction of the accumulation of esterified cholesterol into macrophages, increase of endothelial NO synthetase, reduction of the inflammatory process, increased stability of the atherosclerotic plaques, restoration of platelets activity and of the coagulation process **(Bellosta et al,2000)**

Adverse effects of Statin therapy:

Statins are generally well tolerated. The most important adverse effects are liver and muscle toxicity. Myopathy can happen if inhibitors of cytochrom P450 or other inhibitors of statins metabolism are administered together with statins, determining the increase of their blood concentration. Such are the azole antifungals. **(Maron et al,2000)**

Fibric acid Derivatives :

Role of Transcription Factors in Mediating Fibrate Action:

Fibrates are synthetic ligands for PPAR-α. (**Kliewer et al, 1997, Forman et al, 1997, Devchand et al,1996).** PPAR-α is predominantly expressed in tissues that metabolize high amounts of FAs, such as liver, kidney, heart, and muscle. **(Auboeuf et al, 1997)**

It has been known for several years that fibrates induce peroxisome proliferation in rodents. **(Schoonjans et al,1996)** .This process is linked to the induction of transcription of genes involved in peroxisomal ß-oxidation and is mediated by specific transcription factors, therefore termed peroxisome proliferator-activated receptors (PPARs).

Fibrates are generally effective in lowering elevated plasma triglycerides and cholesterol. The magnitude of lipid changes depends, however, on the patient's pretreatment lipoprotein status **(Tikkanen M , 1992)** as well as the relative potency of

the fibrate use **(Zimetbaum et al,1991).** The most pronounced effects of fibrates are a decrease in plasma triglyceride-rich lipoproteins (TRLs). Levels of LDL cholesterol (LDL-C) generally decrease in individuals with elevated baseline plasma concentrations, and HDL cholesterol (HDL-C) levels are usually increased when baseline plasma concentrations are low**. (Tikkanen , 1992)**

Tolerability and Safety :

In general, fibrates are considered to be well tolerated, with an excellent safety profile. A low incidence of fibrate-associated toxicity has been reported in almost every organ system **(Sgro et al, 1991)**

Members of the 2 most popular classes of lipid-lowering drugs, HMG CoA reductase inhibitors and fibrates, cause cancer in rodents **(Newman et al, 1996).** Although the mechanism may be related to peroxisome proliferation, a definite link has not yet been established. In humans, long-term administration of various fibrates does not cause peroxisome proliferation or any other morphological changes in the liver **(Gariot et al,1983, Angelin et al,1984) .** Extrapolation of this evidence of carcinogenesis from rodents to humans is uncertain.

Clinically relevant interactions of fibrates with other antihyperlipidemic drugs include rhabdomyolysis (reported in combination with HMG CoA reductase inhibitors) and decreased bioavailability when combined with some bile acid sequestrants. Finally, potentiation of the anticoagulant effect of coumarin derivatives may cause bleeding**. (Blum et al, 1992)**

Bile acid binding resins:

Bile acid binding resins are effective for primary and secondary prevention of coronary artery disease**.(Insull, 2006).** Clinical experience indicates that bile acid binding resins can be beneficial for various patients, including different ages and both sexes.

The bile acid binding resins form non-absorbable complexes with bile acids in the gastrointestinal tract and increase their fecal excretion, thereby preventing their reabsorption and removing them from the enterohepatic circulation.

Thus, these agents have a dual effect. They diminish the ability of bile acids to solubilize dietary lipids and stimulate the liver to convert endogenous cholesterol into bile acids in an attempt to maintain bile acid pool size **(Staels et al, 2007)**

CYP7A1 is a cytochrome (CYP) P450 enzyme that catalyzes the hydroxylation of cholesterol at the 7 α position, the classic pathway of bile acid synthesis, and is the major regulatory point in this pathway **(Gilardi et al,2007)**. Bile acid binding resins were initially selected for targeting the negative feedback regulation of CYP7A1 to reduce plasma cholesterol.

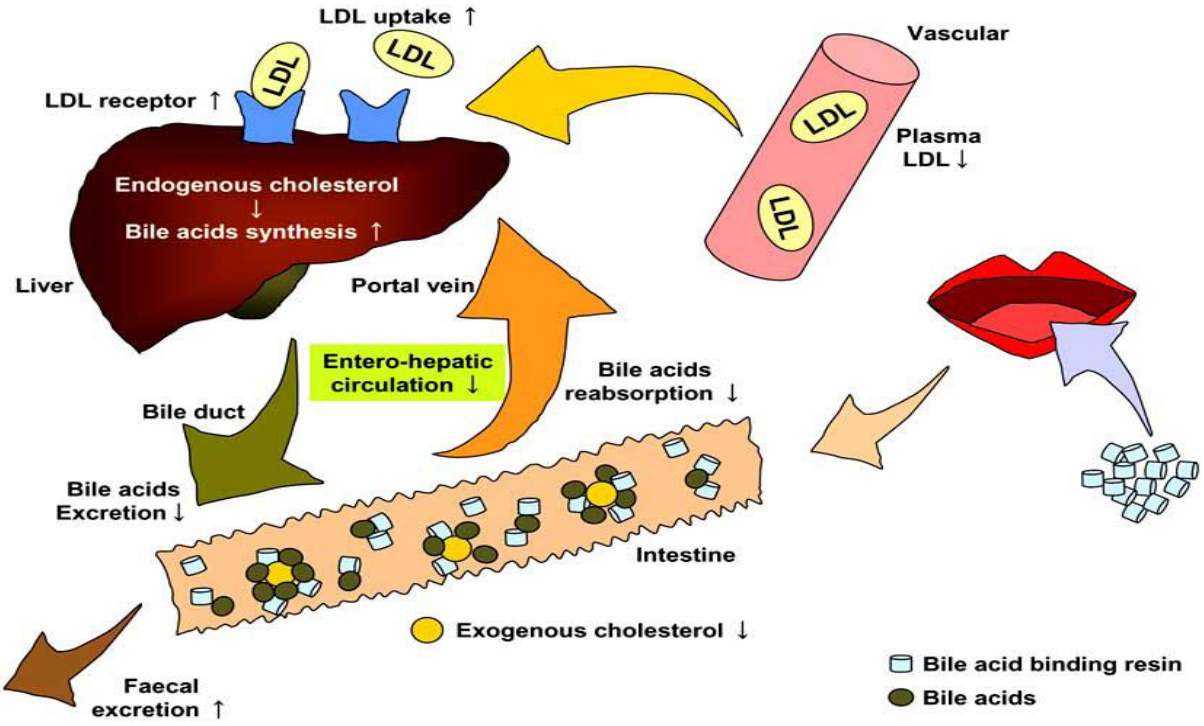

***Figure 4* : Mechanism of bile acid binding resins**

Four bile acid binding resins are currently available: Colestyramine ,Colestipol , Colesevelam HCl and Colestimide

BILE ACID BINDING RESINS: SAFETY AND TOLERABILITY

The major adverse effects associated with colestyramine and colestipol include constipation and flatulence, leading to high patient discontinuation rates, between 40% and 60% **(Andrade SE, et al,1995, Avorn et al, 1998)**

To date, no serious adverse effects of colestimide have been found. However, colestimide is known to cause constipation **(Kajiyama et al ,1996, Suzuki ,2007)**. Some patients treated with colestimide need laxatives, such as magnesium oxide, to prevent constipation. Although these may lead to better patient compliance, bile acid binding resins are contraindicated in patients with bowel obstruction.

Twenty-five years of controlled clinical trials have shown that treating dyslipidemia with bile acid binding resins, alone and in combination with other lipid-lowering drugs such as statins, is both safe and effective**. (Insull , 2006)**

Niacin : (Nicotinic acid)

Nicotinic acid is particularly effective in lowering the blood concentrations of low and very low density lipoprotein cholesterol and in increasing the concentration of high density lipoprotein cholesterol.

Intake at quantities of one gram or more however not only provide pharmacological benefits but also carries significant risk of adverse effect , thus requiring medical supervision and monitoring.

Serious side effects of nicotinic acid have occasionally occurred when gram quantities were taken to lower serum lipids**. (Rader et al ,1992).**

More severe reactions may produce jaundice, fatigue, fulminant liver failure. **(Clementz et al,1989)**

Combination of Drug Tretment in Hyperlipidemia :

The combination of two lipid lowering drugs is very useful in the treatment of severe hyperlipoproteinemia,particularly with heterozygous familial hypercholesterolemia (type II a) in whom single drug therapy often fails to achieve satisfactory plasma levels of LDL-C.

The commonly used drug combinations are as follows :

1.BILE ACID BINDING RESINS plus FIBRATES:

Recommended for familial combined hyperlipidemia.(type II b).

2.BILE ACID BINDING RESINS plus NIACIN :

Effective in familial hypercholesterolaemia (type II a) and also in familial combined hyperlipidemia.(type II b).

3.BILE ACID BINDING RESINS plus STATINS:

Highly effective in reducing LDL-C in patients of familial hypercholesterolaemia (type II a)

4.BILE ACID BINDING RESINS plus NIACIN plus STATIN:

Specially useful in patients with severe disorders due to elevated LDL (type II a & II b)

5.NIACIN plus STATIN:

An efficacious combination for familial combined hyperlipidemia.(type II b) and familial hypercholesterolaemia (type II a).

6.STATINS plus EZETIMIBE:

Synergistic combination for treating primary hypercholesterolaemia and can also be used for the treatment of individuals with homozygous familial hypercholesterolaemia (type II a) **(Sharma et al,2007)**

3.4.2.2 Recent Targets in treatment of Hyperlipidemia:

Patients with familial hypercholesterolemia (FH) are not always able to achieve target levels of low-density lipoprotein (LDL) cholesterol with currently available medications. A number of novel pharmaceutical approaches to LDL cholesterol-lowering have been in development.

Antisense oligonucleotides are molecules that are injected subcutaneously and cause decreased release of apolipoprotein B-containing lipoproteins from the liver. **(Anne , 2010)**

Elevated apoB and LDL-C levels are associated with premature atherosclerosis in several inherited diseases, including familial hypercholesterolemia, familial defective apoB-100, and familial combined hypercholesterolemia **(Kane et al, 2001, Greevenbroek, et al,2002)**

Abnormalities in apoB-100 metabolism that increase the risk for CHD are also observed in diabetes mellitus and obesity **(Chan et al, 2002, Grundy, 1998.).** Conversely, mutations that interfere with apoB synthesis lower plasma levels of apoB and LDL-C and appear to be associated with reduced levels of atherosclerosis (**Linton, et al, 1993, Schonfeld et al, 2003).** These genetic observations have prompted interest in pharmacologic inhibition of apoB synthesis. Regrettably, the potential benefits of directly reducing apoB synthesis via traditional therapeutics have never been explored.

Antisense oligonucleotides (ASOs) are novel therapeutic agents that safely and selectively reduce levels of a specific mRNA**. (Crooke, 2001)** The pharmacokinetics of antisense drugs has been extensively characterized in multiple animal models and in clinical settings **(Graham, et al, 1998, Zhang et al,2000).**

After parenteral administration, first- and second-generation ASOs are widely distributed in tissues; the highest concentrations are found in kidney and liver **(Graham, et al,1998, Zhang et al,2000)** and the drug levels in these organs correlate directly with pharmacological activity in vivo**.(Yu et al, 2001, Zhang et al, 2000)**

Increasing Macrophage Cholesterol Efflux:

Cholesterol efflux, the first step of the RCT pathway, plays a critical role in maintaining intracellular cholesterol homeostasis. Peripheral tissues gain cholesterol via synthetic pathways and direct uptake of circulating lipoproteins but are largely unable to catabolize it. The toxic buildup of cholesterol in arterial foam cells is thought to play a key role in the initiation and progression of atherosclerotic plaque development, and potentially plaque rupture as well. Once assumed to be a largely passive process, recent research has demonstrated that cholesterol efflux occurs largely via ABCA 1(ATP binding cassette A1) and ATP-binding cassette G1 (ABCG1).

The two transporters differ in their acceptor specificities, with ABCA1 responsible for efflux to lipid-poor apoA-I and ABCG1 promoting efflux to mature HDL particles. Animal studies have confirmed that both ABCA1 and ABCG1 facilitate macrophage RCT**(Wang et al,2007)** Mice deficient in both of these proteins exhibit dramatic increases in foam cell accumulation and atherosclerosis, reinforcing the concept that the macrophage cholesterol efflux pathway is antiatherogenic in vivo. **(Yvan-Charvet, et al,2007, Out et al,2008)**

Similarly, loss of function ABCA1 mutations in humans, which underlies Tangier disease, is associated with inability to lipidate apoA-I particles and cholesterol build-up in peripheral tissues **(Rust et al,1999, Bodzioch et al,1999)**

LXR Agonism:

Liver X receptors (LXR), including LXRα and LXRβ, serve as the major regulators of cellular ABCA1 and ABCG1 expression via binding with the heterodimer retinoid X receptor **(Repa et al,2000).** Accordingly, pharmacologic LXR agonism has been shown to promote macrophage RCT and decrease atherosclerosis in mouse models**. (Naik et al , 2006, Terasaka et al, 2003)**

Recent studies have highlighted the primary role of the macrophage in mediating LXR agonists' antiatherogenic activity **(Levin et al,2005, Teupser et al,2008).**

Because LXRα is the predominant subtype in the liver, selective agonism of LXRβ may help overcome the undesirable hepatic effects seen with nonselective stimulation.

Indeed, LXRβ-selective agonists have been developed that retain the ability to promote macrophage cholesterol efflux **(Molteni et al,2007).** As such, LXR agonism remains a highly plausible and conceptually attractive therapeutic target, particularly if it can be accomplished with selective targeting of the macrophage or intestine.

Improving HDL Acceptor Number and Function :

HDL particles are highly heterogenous with regard to size, lipid composition, and protein cargo. Multiple approaches seek to enhance the efflux capacity of circulating plasma in vivo via oral or infusion therapies.

Increased apoA-I Expression :

A large body of evidence suggests that apoA-I, which makes up 70% of HDL protein, is antiatherogenic. Increased apoA-I expression increases plasma HDL levels, promotes RCT, and leads to decreased atherosclerosis in several animal models**. (Zhang et al,2003)**

One study documented atherosclerotic plaque regression, an elusive "holy grail" of modern cardiology, with hepatic apoA-I overexpression**. (Tangirala et al, 1999).** Small molecules that promote hepatic apoA-I expression in humans may recapitulate these strikingly beneficial effects. One such compound, RVX-208 (Resverlogix, Calgary, AB, Canada), recently entered early-phase clinical trials. Serum from human patients treated with RVX- 208 exhibited increased cholesterol efflux capacity despite an only modest increase in HDL-C levels**. (Gordon et al,2009)**

A second mechanism of increasing apoA-I production is via peroxisome proliferator-activated receptor α (PPARα) agonism. Although PPARα stimulation has multiple effects on lipid metabolism, including increasing ABCA1 expression, most promising is its stimulation of apoA-I production **(Duffy et al, 2009).** Although currently prescribed fibrates are week PPARα agonists, several agents with substantially

increased potency and selectivity have been developed. LY518764 (Eli Lilly and Company, Indianapolis, IN) has been shown to increase apoA-I production rate by 31% in patients with metabolic syndrome. **(Millar et al, 2009**)

ApoA-I Mimetic Peptides :

Substantial research has focused on the development of a small mimetic peptide, ideally with oral administration, that can mimic the effects of infusing full-length apoA-I**. (Van Lenten et al,2009)**

One such peptide, D-4F, was engineered to contain only 18 amino acids but retain its lipid-binding properties. Furthermore, the use of only D-amino acids allows the molecule to escape gastrointestinal peptidase breakdown. Oral D-4F was associated with enhanced RCT and decreased atherosclerosis in mouse models**. (Navab et al, 2004)**

Early-phase clinical investigation of single-dose D-4F demonstrated proof of concept with regard to safety, modest oral bioavailability, and a dose-dependent improvement in measures of isolated HDL anti inflammatory activity**.(Bloedon et al,2008)** .The future of D-4F and other apoA-I mimetic peptides will likely depend on improving bioavailability and larger-scale evidence of enhanced HDL functionality.

Cholesteryl Ester Transfer Protein Inhibition :

Patients with genetic cholesteryl ester transfer protein (CETP) deficiency have markedly increased HDL-C levels, a discovery that stimulated efforts to achieve similar effects with pharmacologic therapy**. (Brown et al,1989)** . CETP inhibition therefore represents a strategy of increasing acceptor concentrations for macrophage cholesterol efflux. This concept was reinforced when both genetic deficiency and pharmacologic CETP inhibition were associated with increased cholesterol efflux capacity, primarily via the ABCG1 transporter. **(Matsuura et al, 2006, Yvan-Charvet et al, 2007)**

Despite being an important determinant of plasma HDL levels, the influence of CETP on RCT and cardiovascular disease remains highly controversial **(Tall et al, 2007).** No increase in fecal sterol excretion was noted in a human study of pharmacologic CETP inhibition, although the limitations of the methodology preclude definitive conclusions regarding RCT **(Brousseau et al, 2005).** The field was dealt a major blow when a phase 3 clinical trial of one potent CETP inhibitor, torcetrapib (Pfizer, New York, NY), was terminated after an increase in mortality and cardiovascular events was noted in the treatment group.

This occurred despite the predicted effects on patients' lipid profiles, with a 72% increase in HDL-C and 25% reduction in LDL-C **(Barter et al,2007).** Unfortunately, torcetrapib was linked to off-target elevations in aldosterone levels and blood pressure, potentially explaining a portion of the adverse effects.

Two other CETP inhibitors currently in development, anacetrapib (Merck, Whitehouse Station, NJ) and dalcetrapib (Roche Pharmaceuticals, Basel, Switzerland), show no effects on blood pressure and are likely to provide much-needed insight into the mechanism's utility.

Endothelial Lipase Inhibition :

Endothelial lipase (EL) plays a major role in modulating both HDL particle composition and catabolism **(Jaye et al,1999).** Like other members of the lipoprotein lipase family, EL is a secreted protein that binds to the endothelial surface. It primarily hydrolyzes phospholipids on circulating HDL particles, resulting in accelerated apoA-I catabolism.**(Maugeais et al,2003)** Animal studies have noted substantially increased HDL-C levels and decreased atherosclerosis in EL-deficient mice **(Jin et al,2003).** Decreasing EL activity has thus been proposed as an effective way of increasing plasma levels of HDL acceptors and potentially enhancing RCT.

Microsomal transfer protein inhibitors block the accumulation of triglyceride into apolipoprotein B precursors. **(Anne , 2010)**

Lipoproteins are assembled in the endoplasmic reticulum, matured in the Golgi, and secreted by cells. Their biosynthesis depends on two proteins: apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTP). ApoB is a structural protein.

MTP is an essential chaperone for the assembly of apoB lipoproteins **(Hussainet al, 2008)**. It transfers several lipids including triacylglycerols, phospholipids, and cholesteryl esters. Mechanistic studies revealed that MTP plays a novel role in cholesteryl ester biosynthesis **(Iqbal et al, 2008)** .Triglyceride synthesis involves fatty acid uptake, intracellular transport to microsomes by fatty acid-binding proteins, and acylation with glycerol by several monoacylglycerol and diacylglycerol acyl transferases. Inhibition of these steps will likely reduce cellular triglyceride levels. In this respect, repression of liver fatty acid-binding protein along with MTP inhibition has been shown to lessen steatosis **(Spann et al,2006)**

Several studies have shown that flavonoids inhibit triglyceride transfer activity of MTP **(Allister et al,2005)** .These compounds affect several other biological pathways and have pleiotropic effects. For example, taxifolin, a plant flavonoid, inhibits triglyceride synthesis and MTP activity without increasing cellular lipids. **(Casaschi et al, 2004).** Therefore, joint inhibition of triglyceride synthesis and MTP activity might avoid triglyceride accumulation.

Squalene synthase inhibitors partially block a late step in cholesterol biosynthesis. **(Anne ,2010)**

Squalene synthase (SS) is the first enzyme in the hepatic cholesterol biosynthetic pathway which produces a metabolite (lanosterol) committed to cholesterol synthesis. The result of inhibition of SS is analogous to that of HMG-CoA reductase. Induction of LDL receptors occurs, leading to a decrease in circulating LDL. Thus SS inhibitors act similar to statins, albeit at a different and a more specific level.

Thyroid hormone analogues lower LDL cholesterol and other lipoproteins by a selective effect on certain thyroid hormone receptors, avoiding the adverse effects of excessive thyroid hormone levels. Several of these classes of lipid-modifying agents are currently in clinical trials. Long-term safety data will be needed before any are available to be used clinically, but some hold significant potential for improving treatment options for patients with FH. **(Anne, 2010)**

Other therapeutic targets in lipid metabolism:

Guggulsterone (gugulipid) is a widely used yet controversial hypolipidemic drug. The plant sterols E- and Z-guggulsterone are the active compounds **(Chiang et al, 2004)** derived from the gum resin of the mukul myrrh tree (Commiphora mukul). They act as antihyperlipidemic agents via the antagonism of the Farnesoid X receptor (FXR) **(Urizar et al 2002, Deng, 2007)** and up-regulation of the bile salt export pump (BSEP). **(Szapary et al, 2003).** E- and Z-guggulsterone are currently marketed under the brand name Gugulipid.

Most of the clinical trials conducted in Asia demonstrated hypolipidemic activity of gugulipid with an average decrease of 10%-30% and 10%-20% in total cholesterol and triglyceride respectively. **(Szapary et al, 2003)**

Ezetimibe blocks uptake of cholesterol into jejuna enterocytes, hence selectively blocking dietary and biliary cholesterol absorption from the gut. It acts by decreasing the intestinal cholesterol supply to the liver, lowering hepatic cholesterol levels and thus inducing LDL receptor expression.

Unlike the bile acid sequestrants, it does not interfere with absorption of fat-soluble drugs. Moreover, ezetimibe does not increase serum TG**. (Charlton et al, 2007).**

3.5 Animal model in the study:

In present study, acute hyperlipidemia was induced in rats using Triton WR 1339.

Synonyms - Triton W.R.1339, Tyloxapol, Tyloxapolum (Latin), Tyloxypal , Tiloxapol (Spanish)

IUPAC Name - formaldehyde; oxirane; 4-(2,4,4-trimethylpentan-2-yl) phenol

Chemical Formula - $C_{17}H_{28}O_3$

The use of triton WR 1339 induced hyperlipidaemia as an important approach to screen the action of hypolipidemic drugs. **(Paoletti , 1962).** Triton WR-1339-induced hyperlipidemic rats are a globally accepted model used to evaluate potential hypolipidemic drugs **(Otway et al,1967)** .

This model is widely used for a number of different aims, **(Ye-Yun et al,2005)** particularly, in rats it has been used for screening natural or chemical hypolipidemic drugs **(Harbowy et al,1997)**. It is a non-anionic detergent of polymeric3 structure that has been successfully used in several studies to induce hypercholesterolemia. **(Kourounakis et al, 2002, Okunevich et al,2002)**

Mechanisms responsible for producing hyperlipidemia by Triton WR-1339 :

- It is convenient to use a T-WR induced hyperlipidemia model, because it can produce dose-related hyperlipidemia in a short period.
- The nonionic surfactant, T-WR, has been used in lipid metabolism research to inhibit the removal of lipoprotein from the circulation.**(Borensztajn et al, 1976)** .
- Triton WR 1339 is administered i.v. or i.p. in rodents to produce hypercholesterolemia by accelerating hepatic cholesterol synthesis. (**Frantz et al, 1955)**
- Triton WR-1339 is a nonionic detergent that prevents catabolism of triacylglycerol-rich lipoproteins by lipo-protein lipase and is commonly used for *in vivo* determination of triacylglycerol production, and very low density lipoprotein (VLDL) secretion or clearance rate. **(Schurr et al, 1972, Hayashi et al, 1981)**

In the present study, Hyperlipidemia was induced in wistar albino rats by single intraperitoneal administration of Triton WR 1339 (300 mg/kg) in 0.9 % Nacl solution.

The large decrease in plasma HDL-C levels due to Triton WR-1339 injection results mostly from a progressive displacement of the apo A-1 protein from the HDL surface, without loss of lipid **(Yamamoto et al, 1984)**. Mean while the large increase in plasma TG levels due to Triton administration results mostly from an increase of very low-density lipoprotein (VLDL) secretion by the liver accompanied by strong reduction of VLDL and LDL catabolism.
(Otway et al, 1967).

3.6 General introduction to Oil :

3.6.1 Analytical Parameters for Oils & Fats :

The properties of oils & fats vary along with the degree of unsaturation, average molecular weight & also acidity from hydrolysis.

A no. of parameters are used for their analysis which are included under physical constant & chemical constants.

Physical constants include viscosity, specific gravity, refractive index, solidification point,etc. Under the group of chemical constants, conventionally the parameters like iodine value, acid value, peroxide value, saponification value, unsaponification are noted.**(Kokate et al, 2005)**

1.Acid Value :

It is defined as the number of milligrams of potassium hydroxide required to neutralise the free acids present in 1 g sample of fat or oil.

Generally, rancidity causes free fatty acids liberation, hence acid value is used as an indication of rancid state. **(Kokate et al, 2005)**

2.Saponification Value :

It is defined as the number of milligrams of potassium hydroxide required to neutralise the fatty acids resulting from complete hydrolysis of 1 g of sample of oil or fat.

Saponification value occurs in an inverse proportion to the average molecular weight of fatty acid present in the oil. **(Kokate et al, 2005)**

3.Ester Value :

It is defined as the number of milligrams of potassium hydroxide required to combine with fatty acids which are present in glyceride form in 1 g sample of oil or fat.

Difference between saponification value & acid value is ester value. **(Kokate et al, 2005)**

3.7 Brief information of Safflower oil :

Safflower oil is belonging to the class of Semi-drying oils.

Biological Source: It is a fixed oil obtained from the ripe & dry seeds of *Carthamus tinctorius* Linn

Family : Compositae.

Method of preparation :

For expression of oil, the seeds from promising varities in India namely A-300,S-7-13-3 are selected, cleaned and further processed. About 1000 seeds of safflower weigh 20 to 50 g .The seed normally contain 35 to 38 % of fixed oil.The oil is prepared by expression in expellers or with the help of hydraulic presses. The oil is filtered and further purified. The seed meal or round seeds are subjected to cooking by means of open steam, which ensures maximum yield of oil .The filtered and decolourized oil is packed into suitable containers.

Description :

It is a clear, faint yellowish liquid with characteristic odour and taste. The oil thickens and becomes rancid on exposure to air.

Solubility :

Safflower oil is slightly soluble in alcohol and freely soluble in ether, chloroform, benzene and petroleum-ether.

Standards :

- Specific gravity :- 0.9211 to 0.9215
- Acid value :- 01 – 9
- Refractive index :- 1.472 to 1.475
- Sap. value :- 188 – 194
- Iodine value :- 140 – 150

- Hydroxyl value :- 2.9 – 6.0
- Unsaponifiable fraction :- not less than 1.5
- Thiocyanogen value :- 82.5 – 86.

Chemical Constituents :

SO is belonging to the class of Semi-drying oils & it contains glycerides of palmitic (6.5%), stearic (3.0 %), arachidic (0.296 %), oleic (13.0 %), linoleic (76-79 %) and linolenic acids (90.15 %). The polyunsaturated fatty acid content of the SO is highest (75%) and is said to responsible to control cholesterol level in blood and thereby reduce incidence of heart attacks **(Kokate et al, 2005).**

Mechanism of Action :

- There is the greater diet-induce thermogenesis and the higher fat oxidation rate in rats fed the safflower oil diet compared to those fed the beef tallow diet at least partly accounted for the lower body fat accumulation in the former.
- Consumption of the safflower oil diet elevates the fat oxidation rate is also in good accordance with the observation that the polyunsaturated: saturated ratio of dietary fat influences energy substrate utilization in humans**.(Jone et al, 1988)**
- A diet rich in polyunsaturated fatty acids appears to result in preferential stimulation of the thermogenic activity of brown adipose tissue**. (Merce et al ,1987)**
- High polyunsaturated fat diet could increase solubility of lipids in the circulating lipoproteins and enhance the substrate-enzyme contact by increasing the unsaturation of the lipids**.**
- Thus, Intake of the safflower oil diet may have facilitated the interaction of the lipoprotein triacylglycerol with LPL, resulting in a more rapid removal of the triacylglycerol from blood. **(Engelbergh , 1966)**

Uses : (Kokate et al, 2005).

- It helps to reduce triglycerides, and lower blood pressure.
- The edible oil is used in the manufacture of oleomargarine, as a dietary supplement in hypercholesteremia & also in the treatment of atherosclerosis .
- Due to its high linoleic acid content, it is consumed for the preparation of vegetable ghee.
- Industrially, it is used for preparation of soft soap & water proofing material.

3.8 Brief information of Rice bran oil :

Rice bran oil is belonging to the class of Non-drying-drying oils.**(Kokate et al, 2005)**

Biological source : Rice bran is the cuticle existing between the rice & the husk of the paddy & consists of embryo (germ) & endosperm of the seeds of *Oryza sativa* .
Family: Gramineae. .**(Kokate et al, 2005)**

It is obtained as a byproduct in rice mill during polishing of rice obtained after dehusking of paddy.It contains about 15 % of fixed oil & is obtained by solvent extraction method.

Method of preparation : .**(Kokate et al, 2005)**

The quality of rice bran oil depends upon the time which elapse between milling of the rice & removal of the oil from the bran.

Rice bran contains an active enzyme lipase ,which raises the free fatty acid on storage. The oil obtained from the fresh bran is of good quality & has good flavour & low free fatty acid content.

Therefore, solvent extraction plant for rice bran oil should be set as nearer as possible to the rice milling so as to process out the rice bran oil quickly.

Rice bran is found in extremely small pieces. It is impermeable to solvents. Before solvent extraction, it is subjected to drying, cooking & flaking operations. The normal percolation method of solvent extraction does not serve the purpose with this type of material,but it is pressed & then extracted with solvent special continuous immersion extractors.

Description :

It is a golden yellow oil difficult to bleach, and not affected by temporary heating to 160 ˚ C.

Solubility : It is insoluble in water but soluble in common fat solvents.

Standards :

- Acid value :- 04 – 05
- Saponification value :- 181 – 189
- Iodine Value :- 99 – 108
- Thoicyanogen value :- 69 – 76
- Hydroxyl value :- 05 – 14
- Refractive index :- 1.470 – 1.473
- Specific gravity :- 0.916 – 0.921

Chemical Constituents :

- It contains mainly oleic acid (38.4%), linoleic acid (34.4%) and α-linolenic acid (2.2%) as unsaturated fatty acids, and palmitic (21.5%) and stearic (2.9%) acids as saturated fatty acids In contrast to most common refined vegetable oils, crude rice bran oil contains a rich unsaponifiable fraction (up to 5%) mainly composed by sterols (43%), triterpene alcohols (28%) 4-methyl-sterols (10%) and less polar components (19%) **(Sayre et al. 1990).**
- Phytosterols include β-sitosterol (900 mg%), campesterol (500 mg%), stigmasterol (250 mg%), squalene (320 mg%) and γgamma-oryzanol (1.6%). The so-called gamma-oryzanol, often identified as the active molecule of rice bran oil, is a mixture of ferulic acid esters of triterpene alcohols such as cycloartenol (106 mg%) and 24-methylene cycloartanyl (494 mg%) **(Metwally et al. 1974, Norton, 1995),** firstly isolated by Kaneko and Tsuchiya in the early 1950s **(Kaneko et al.1955).**
- Its fundamental molecular structure is the ferulic acid aromatic phenolic nucleus esterified to cyclopentanperihydrophenanthrene **(Seetharamaiah et al. 1986).**

Rice bran oil contains a little variable quantity of tocotrienols (from 72 to 612 ppm, especially β and γ-tocotrienols), but it is naturally very rich in tocopherol (ca. 100 mg%), similarly to another vegetable oil with well-known antihyperlipidemic action, the soybean oil **(Rukmini et al. 1991, Rogers et al. 1993)**

Mechanism of Action :

- Its specific content of polyphenols (gamma-oryzanol), phytosterols, tocopherols and tocotrienols is supposed to contribute to antihyperlipidemic action, while the particular fatty acid mono- and polyunsaturated composition seems not to be fundamental in its activity **(Rong et al.1992).**
- Possible fundamental antiatherosclerotic role rest on gamma-oryzanol **(Kanbara et al. 1992)**
- Rice bran oil and its unsaponifiable matter significantly increase the faecal excretion of acid and neutral sterols **(Seetharamaiah et al. 1989).**
- It is possible that gamma-oryzanol's antihypercholesterolemic effect is partially due to its sterol moiety, which is partly split off from the ferulic acid part in the small intestine by cholesterol esterase **(Swell et al. 1954, Sugano et al.1997).**
- Rice bran oil's antiatherogenic action could also be based on other mechanisms, for example cholesterol-esterase inhibition by cycloartenol, or by the inhibition of the accumulation within macrophages of cholesterol-esters or by the modulation of cholesterol acid esterase and acyl-CoA-cholesterol-acyltransferase by gamma-oryzanol **(Rukmini et al. 1991).**
- RBO also contains phytosterols which act at the intestinal level by interfering with the absorption of the cholesterol from the gut.**(Narasinga , 2000)**
- Cycloartenol, a triterpene alcohol, has a structure that is similar to cholesterol & it may compete with the binding sites of cholesterol, sequestering cholesterol from the system.
- Tocotrienols are thought to inhibit the HMG Co A reductase activity in the biosynthetic pathway of cholesterol & also possess antioxidant, antithrombotic, anticarcinogenic properties**. (Raghuram et al ,1989)**

Uses:

- Beside the antihyperlipidemic action, the strong antioxidant property of gamma-oryzanol **(Tajima et al.1983, Kim et al.1995, Hiramitsu et al.1991)** and other rice bran oil components, such as tocotrienols and tocopherols may contribute to rice bran oil's antiatherogenic effect.
- One of the most investigated properties of gamma-oryzanol is its anti-ulcerogenic property **(Mizuta et al.1978, Ishihara et al.1984).**
- Gamma-oryzanol can be a LH, TSH, GH and PRL secretion inhibitor. The inhibition of the LH secretion after a single intravenous injection was significantly stronger than PRL's one in normal male rats and ovariectomized female rats **(Yamauchi et al.1980).**
- Fermented rice bran has a significant anti-stress and anti-fatigue effect**. (Kim et al.2002)**
- It is currently used as an edible oil in several countries. It is used in the preparation of vegetable ghee. **(Kokate et al, 2005)**

4. MATERIALS AND METHODS

4.1 Oil Materials & Standard drug

In the present study, Safflower oil (SO), Rice bran oil (RBO) were used as a test drugs and atorvastatin sodium was used as a standard drug. Safflower oil & Rice bran oil were purchased from the S.K Oil industries, Jalgaon, Maharashtra, India, A.P Organics Ltd, Dhuri (Punjab), India respectively. Standard drug atorvastatin sodium was obtained as a gift sample from Torrent Research Center, Gandhinagar, Gujarat, India.

4.2 Chemical test for identification of fixed oil

SO and RBO are fixed oils.

Fixed oils were confirmed by chemical test for glycerine, which was produced by their hydrolysis.

1. Using Sodium Hydroxide: 1 ml 1 % copper sulphate solution & 5 drops of the test oil (SO/ RBO) were mixed. Then 5 drops of 10 % sodium hydroxide solution was mixed. A clear blue solution was obtained which shows glycerine is present in the sample. The cupric hydroxide formed in the reaction does not precipitate out as it is soluble in glycerine.

2. Using Sodium Hydrogen Sulphate : 5 drops of the test oil (SO/ RBO) was taken in a test tube & pinch of sodium hydrogen sulphate was added. Pungent odour emanates from the tube indicating presence of glycerine in the sample. The pungent odour is due to the formation of acrolein. **(Kokate et al, 2005)**

4.3 Evaluation of Physicochemical parameters of SO and RBO

4.3.1 Density: (Martin et al,1991)

Empty density bottle was weighed, 10 ml of the test oil (SO/ RBO) was added in the density bottle, the density bottle was again weighed, weight of 10 ml of test oil (SO/ RBO) was found out. The density of oil was calculated by the following formula,

Density of oil = weight of 10 ml of oil / vol. of oil (10 ml)

4.3.2 Viscosity : (Martin et al,1991)

It was measured with the help of Brookfield Viscometer. For this, following steps were done. The instrument was switched on, Spindle was removed. Automatically the instrument was switched over to calibration mode. Appropriate spindle was attached to spindle hook. Sample container was attached and 500 ml of the test oil (SO/ RBO) was poured. Spindle number (S 61) was entered through spindle key. The desired RPM (600 RPM) was entered through the speed key. The viscosity in centipoises was noted down. % Torque (60.5 %) and temperature was also noted. RPM was increased till we got torque nearly equal to 60.5 % and viscosity in cps. was noted down.

4.3.3 Boiling Point : (Goyal et al, 2009)

Fusion tube was taken, small amount of the test oil (SO/ RBO) was added in it, capillary tube was taken & it was sealed at on end .Now, capillary tube was placed inside the fusion tube in such a manner that open end of capillary tube was dipped into the test oil (SO/ RBO) (i.e in inverted position). Now, fusion tube was attached to the thermometer & then it was placed in oil bath whose temperature was continuously raised by 1 to 2 ˚ C / minute. Temperature was noted at which continuous bubbling started in the fusion tube.

4.3.4 Acid value : (Indian Pharmacopoeia 2007, Volume 1)

Accurately weighed 10 g of the test oil (SO/ RBO) under examination was dissolved in 50 ml of a mixture of equal volumes of ethanol (95 per cent) and ether, which was previously neutralised with 0.1 M potassium hydroxide to phenolphthalein solution. 1 ml of phenolphthalein solution was added and titrated with 0.1 M potassium hydroxide until the solution remains faintly pink after shaking for 30 seconds.

Acid value = 5.61 n/w

Where, n = the number of ml of 0.1 M potassium hydroxide required;

w = the weight, in g, of the substance.

4.3.5 Saponification value : (Indian Pharmacopoeia 2007, Volume 1)

Accurately weighed 2 g of the test oil (SO/ RBO) under examination was introduced into a 200-ml flask of borosilicate glass fitted with a reflux condenser. 25.0 ml of 0.5 M ethanolic potassium hydroxide and a little pumice powder was added and boiled

under reflux on a water-bath for 30 minutes. 1 ml of phenolphthalein solution was added and was immediately titrated with 0.5 M hydrochloric acid (a ml). A blank determination was performed omitting the substance under examination (b ml).

Saponification value = 28.05 (b - a)/w

Where, w = weight, in g, of the substance.

4.3.6 Ester value : (Indian Pharmacopoeia 2007, Volume 1)

The acid value and the saponification value of the test oil (SO/ RBO) was determined.

Ester value = Saponification value - Acid value.

4.4 Experimental Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute Of Pharmacy, Nirma University, Ahmedabad as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Protocol number is **IPS/PCOL/MPH10-11/004** dated 9 th August, 2010.

Healthy adult Wistar rats of either sex (250-300 gm weight) were selected for the study. Animals were maintained at 22 ± 2^{o}C and kept in well ventilated animal house under natural photoperiodic condition in polypropylene cages with free access to food and water *ad libitum*. During the period of experiment the animals were fed with the Amrut Rat Diet supplied by Pranav Agro Industry Ltd, Pune, India. Animals were acclimatized for one week before starting the experiment.

4.5 Triton-WR 1339 Induced Hyperlipidemia

Experimental design:

Rats (n=48) were randomized into the eight groups as mentioned below:-

Group 1 (Normal Control) : They served as a normal control group.

Group 2 (Triton WR 1339 Control) : On 31st day intraperitoneal injection of triton WR 1339 (300 mg/kg in saline) was given.

Group 3 (Triton WR 1339 + SO) : Treatment was given with SO (7 ml/kg, orally) for 30 days & on 31st day, intraperitoneal injection of triton WR 1339 (300 mg/kg in saline) was given.

Group 4 (Triton WR 1339 + RBO) : Treatment was given with RBO (7 ml/kg, orally) for 30 days and on 31st day, intraperitoneal injection of triton WR 1339 (300 mg/kg, in saline) was given.

Group 5 (Triton WR 1339 + (SO + RBO, 2:8)) : Treatment was given with combination of SO + RBO (2:8, 7 ml/kg, orally) for 30 days and on 31st day, intraperitoneal injection of triton WR 1339 (300 mg/kg in saline) was given.

Group 6 (Triton WR 1339 + (SO + RBO , 3:7)) : Treatment was given with combination of SO + RBO (3:7, 7 ml/kg, orally) for 30 days and on 31st day, intraperitoneal injection of triton WR 1339 (300 mg/kg in saline) was given.

Group 7 (Triton WR 1339 + (SO + RBO , 5:5)) : Treatment was given with combination of SO + RBO (5:5, 7 ml/kg, orally) for 30 days and on 31st day, intraperitoneal injection of triton WR 1339 (300 mg/kg in saline) was given.

Group 8 (Triton WR 1339 + Atorvastatin) : Treatment was given with standard drug atorvastatin (2 mg/kg in 0.5 % CMC suspension) for 30 days and on 31st day, intraperitoneal injection of triton WR 1339 (300 mg/kg in saline) was given.

After 24 hours of triton injection, the blood was collected from the retro- orbital plexus of the rat for the estimation of lipid profile and animals were sacrificed using excess dose of ether. The liver was isolated and stored in a deep freezer at -20 ˚ C temperature for estimation of oxidative stress parameters.

4.5.1 Parameters Assessed:

4.5.1.1 Lipid profile in serum:

- Total cholesterol
- Triglycerides
- HDL cholesterol
- LDL cholesterol

4.5.1.2 Oxidative stress parameters in liver homogenate:

- Total Protein
- Pro- oxidant :- Malondialdehyde.(MDA)
- Anti-oxidant :- Reduced Glutathione (GSH)

4.6 MEASUREMENT OF VARIOUS PARAMETERS

4.6.1 Estimation of lipid profile in serum :

4.6.1.1 Collection of serum :

The blood samples were withdrawn from retro-orbital plexus under light ether anaesthesia without any anticoagulant and allowed to clot for 10 minutes at room temperature. It was centrifuged at 2500 RPM for 20 minutes. The serum obtained was kept at 4ºC until used.

4.6.1.2 Estimation of total cholesterol :

In vitro quantitative determination of the activity of cholesterol in serum was done using enzymatic kit **(Lab Care Diagnostics, India Limited).**

Principle

Cholesterol esters are hydrolyzed by Cholesterol esterase to produce cholesterol. Hydrogen Peroxide is then produced from oxidation of cholesterol by cholesterol oxidase. The indicator quinoneimine is formed from hydrogen peroxide and 4 - aminoantipyrine in the presence of phenol and peroxide. The absorption of the red quinoneimine dye is proportional to the concentration of cholesterol in the sample.

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and sample as shown below.

	Blank	Standard	Sample
Sample	-	-	10 µl
Standard	-	10 µl	-
Reagent	1000 µl	1000 µl	1000 µl

Mixed and incubated for 5 mins at 37°C (or 10 mins at 20 - 25°C). The absorbance of the sample (AT) and standard (AS) against reagent blank was measured at 505 nm. The colour was stable for 30 mins at 20 - 25°C.

Calculations:-

AT/AS x Conc. Standard = mg/dl Total Cholesterol

4.6.1.3 Estimation of triglycerides:

In vitro quantitative measurement of triglyceride (neutral fat) concentration in serum was done by using kit **(Lab Care Diagnostics, India Limited)**.

Principle

Triglycerides are determined after enzymatic hydrolysis with lipases. The quinonemine indicator is formed from hydrogen peroxide, 4- aminophenazone, and 4-chlorophenol under the catalytic influence of peroxidase.

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Sample as shown below:-

	Blank	Standard	Sample
Sample	-	-	10 µl
Standard	-	10 µl	-
Reagent	1000 µl	1000 µl	1000 µl

Mixed and incubated for 5 mins at 37°C (or 10 mins at 20 - 25°C). The absorbance of the sample (AT) and standard (AS) against reagent blank was measured at 505 nm. The colour was stable for 30 mins at 20 - 25°C.

Calculations:- AT/AS x Conc. Std. = mg/dl Triglycerides

4.6.1.4 Estimation of LDL Cholesterol :

In vitro quantitative measurement of LDL-C concentration in serum was done by using kit (**Lab Care Diagnostics, India Limited.)**

Principle

Direct determination of serum LDL (low-density lipoprotein cholesterol) levels without the need for any pre-treatment or centrifugation steps.

The assay takes place in two steps.

1. Elimination of lipoprotein non-LDL.

CHE

Cholesterol esters ⟶ Cholesterol + Fatty acids

CHOD

Cholesterol + O_2 ⟶ 4-Cholestenone + H_2O_2

Catalase

$2H_2O_2$ ⟶ $2H_2O+O_2$

2. Measurement of LDLc :-

CHE

Cholesterol esters ⟶ Cholesterol + Fatty acids

CHOD

Cholesterol + O_2 ⟶ 4-Cholestenone + H_2O_2

POD

$2H_2O_2$ + TOOS + 4-AA ⟶ $2H_2O+O_2$

The Intensity of the color formed is proportional to the LDL concentration in the sample.

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Sample as shown below.

	Blank	Standard	Sample
R1 (µl)	750	750	750
Standard (µl)	-	10	-
Sample (µl)	-	-	10
Mix and incubate for 5 mins at 37°C.			
R2 (µl)	250	250	250

Mixed and incubated for 5 mins at 37°C. The absorbance (A) was measured against the Blank at 546 nm.

Calculation:

$$\frac{\text{A Sample}}{\text{A Calibrator}} \times \text{Calibrator conc.} = \text{mg/dL of LDLc in the sample}$$

Concentration of Calibrator is 56 mg/dl.

4.6.1.5 Estimation of HDL Cholesterol :

In vitro quantitative measurement of HDL-C concentration in serum was done by using kit (**Lab Care Diagnostics, India Limited)**

Principle

Direct determination of serum HDL (high-density lipoprotein cholesterol) levels without the need for any pre-treatment or centrifugation of the sample. The method depends on the properties of a detergent which solubillizes only the HDL so that HDL-c is released to react with the cholesterol esterase, cholesterol oxidase and chromogens to give colour. The non HDL lipoprotein LDL, VLDL and chylomicrons are inhibited from reacting with the enzymes due to absorption of the detergents on their surfaces. The intensity of the color formed is proportional to the HDL concentration in the sample

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and sample as shown below:-

	Blank	Standard	Sample
R1 (µl)	750	750	750
Standard (µl)	-	10	-
Sample (µl)	-	-	10
Mix and incubate for 5 mins at 37°C. Read absorbance A1.			
R2 (µl)	250	250	250

Mixed and incubated for 5 min at 37^{o}C. The absorbance A2 was measured against blank at wavelength 600 nm. The increase of the absorbance ΔA=A2-A1 was calculated.

Calculations:

HDL-C (mg/dl) = ΔA sample / ΔA Calibrator X Calibrator conc.

4.6.1.6 Estimation of VLDL Cholesterol : (Russell et al,1990)

Estimation of VLDL-cholesterol was done using the Friedwald's formula.

VLDL cholesterol = Triglycerides / 5.

4.6.1.7 Measurement of various coronary disease risk factors :

- Atherosclerotic Index (A.I. = LDL-C / HDL-C) **(Bhandari et al , 2008)**
- Ratio of HDL/TC

4.6.2 Estimation of oxidative stress parameters in liver :

4.6.2.1 Isolation of liver :

All the animals were euthanasiously sacrificed by the excess dose of ether. Liver was collected and was blotted free of blood and tissue fluids. Then it was weighed on balance and the weight was noted down. The liver was stored in a deep freezer at -20 ˚ C temperature for estimation of oxidative stress parameters.

4.6.2.2 Preparation of the tissue homogenate :

Liver, kept in cold conditions (pre-cooled in inverted petridish on ice) was removed. It was cross chopped with surgical scalpel into fine slices and was chilled in the cold 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10% w/v) with 25 strokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for enzymes assays.

4.6.3 Measurement of oxidative stress parameters :

4.6.3.1 Total Protein estimation:

Total Protein was estimated by the method of **Lowry et al, 1951.**

Principle :

The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple color complex , with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins.

Reagents :

1. Reagent A : 2 % sodium carbonate in 0.1 N NaOH.
2. Reagent B: 0.5 % copper sulphate ($CuSO_4$. 5 H_2O) in 1 % potassium sodium tartrate.
3. Reagent C : Alkaline copper solution (Mix 50 ml of solution A & 1 ml of solution B prior to use)
4.Reagent D : Folin- Ciocalteau reagent.

Procedure :

Following steps were performed for the test.

Blank	**Test**
0.2 ml of D.W.	0.2 ml of Homogenate
Diluted upto 1 ml with Tris HCL	Diluted upto 1 ml with Tris HCL
5 ml Reagent C	5 ml Reagent C
Allowed it for 10 minutes	
0.5 ml Reagent D	0.5 ml Reagent D

Mixed well and kept for 20 minutes at room temperature. The absorbance was measured against blank at 600 nm using spectrophotometer.

Calculation:-

Y = 0.0012X + 0.0673

Where, **X** = Conc.of protein

Y = Absorbance of test sample

Units: µg protein / mg wet tissue.

4.6.3.2 Lipid peroxidation:- (MDA) :

Malondialdehyde (MDA) was estimated by the method of **Slater et al., 1979.**

Principle:

The method estimates malondialdehyde (MDA), a product of lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured colorimetrically at 535 nm.

Reagents:

1. **Sodium lauryl sulphate (SLS) (8%) :** 8 gm of SLS in 100 ml of distilled water.

2. **Acetic acid (20 %) :** Prepared in 0.27 M hydrochloric acid (2.29 ml HCL in 100 ml water)

3.**Thiobarbituric acid(TBA) (1% in Tris hydrochloride, pH 7): (Freshly prepared):-** 1 gm of thiobarbituric acid in 100 ml of Tris hydrochloride buffer pH 7.

4.**Trichloroacetic acid (TCA) (10%) :** 10 gm of trichloroacetic acid in 100 ml of distilled water.

Procedure:

Following steps were performed for the test.

Blank	**Test**
0.2 ml of D.W.	0.2 ml of Homogenate
0.2 ml of SLS	0.2 ml of SLS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml DW	0.6 ml DW
Heated for 45 min in water bath at 95^0C and cool	
2 ml mixture + 2 ml TCA	2 ml mixture + 2 ml TCA
Centrifuge on 1000 RPM for 5 min	

The absorbance of the developed pink colour was measured at 532 nm.

Calculation:

A = a * b * c

A = Absorbance of test sample

a = Molecular Extinction coefficient ($1.56 * 10^5$ cm^{-1})

b = Path length (1 cm)

c = Conc. of sample

Units:- nanomoles of MDA / mg of protein

4.6.3.3 Reduced Gluathione :

Reduced of glutathione (GSH) was estimated by the method of **Moran et al , 1979.**

Principle :

Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), A disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which measured colorimetrically at 412 nm.

Reagents :

1. Trichloroacetic acid (TCA) (10%) : 10 gm of TCA in 100 ml of distilled water.

2. 0.3 M Na_2HPO_4 : 4.26 gm of Na_2HPO_4 in distilled water.

3. DTNB (Dithiobis nitro benzoic acid) (Fresh) : 40 mg in 100 ml of 1% Sodium citrate & cover with aluminium foil.

Procedure:

Blank	**Test**
0.2 ml of D.W.	0.2 ml of Homogenate
1 ml of TCA (10%)	1 ml of TCA (10%)
Keep in ice bath for 30 min & Centrifuge for 10 min at 4 ˚C at 3000 RPM, take 0.5 ml of supernatant	
0.5 ml of Supernatant	0.5 ml of Supernatant
2 ml di-sodium hydrogen phosphate	2 ml di- sodium hydrogen phosphate
0.25 ml DTNB(Cover with aluminium foil)	0.25 ml DTNB(Cover with aluminium foil)

Mixed well and then the absorbance was measured against blank at 412 nm using spectrophotometer.

Calculation: Y = 0.015X - 0.0265

Where, **X** = Conc. of reduced of glutathione**.**

Y = Absorbance of test sample, **Units:** µg of GSH / mg of protein.

4.7 STATISTICAL ANALYSIS

All the values are expressed as Mean ± S.E.M. Statistics was applied using GraphPad Prism 5.0 version. Statistical significance between all the groups was carried out using one way ANOVA analysis followed by Tukey's multiple comparision test. Differences were considered to be statistically significant when $p < 0.05$.

6. DISCUSSION:

Mortality from cardiovascular disease is the leading cause of death in the industrialized world. For several decades, hypercholesterolemia has been regarded as a major risk factor in the development of CVS disorder like coronary artery disease **(Wald et al, 1995, Law et al, 1994).** Hyperlipidemia is a metabolic disorder, specially characterized by alteration occurring in serum lipid and lipoprotein profile due to increased concentration of Total Cholesterol (TC) , Low Density Lipoporotein cholesterol (LDL-C), Very Low Density Lipoprotein Cholesterol (VLDL-C) and Triglyceride (TG) with a concominant decrease in the concentration of High Density Lipoprotein Cholesterol (HDL-C) in the blood circulation **(Dhuley et al, 1999).**

Cholesterol is essential for the growth and viability of eukaryotic cells, however, the unregulated cellular accumulation of cholesterol predisposes atherosclerosis. Cellular cholesterol homeostasis remains crucial for the prevention of cardiovascular disease **(Bocan et al, 1998, Hay et al, 1999).** Moreover, an increased serum LDL-C has been shown to be a strong, consistent, and independent risk factor for coronary heart disease (CHD) in western populations **(Grundy et al, 1990).** Oxidation of LDL-C is traditionally accepted as initiating processes leading to the development of atherosclerosis. The earliest events in the development of the pathology of atherosclerosis are endothelial dysfunction and oxidative stress in the vascular cell wall, activation of inflammatory cells and migration of vascular smooth muscle cells to the intima with the modification of the extracellular matrix, leading to the artery remodelling **(Ross, 1999).**

Hypertriglyceridemia is associated with increased risk of coronary disease. VLDL and IDL have been found in atherosclerotic plaques. Acute pancreatitis is one of the major clinical sequelae of hyperlipidemias which occurs in patient with marked hypertriglyceridemia. **(Katzung B.G, 2007)**

HDL-C levels have been recognized as a strong inverse predictor of cardiovascular risk since the 1970s **(Miller et al, 1975).** Population-based studies suggest that an

increment of even 1 mg/dL in HDL-C is associated with a 3–4% reduction in mortality from cardiovascular disease. **(Gordon, 1989)**

Development of atherosclerosis is thought to be closely dependent upon increased oxidative stress, that is, an imbalance between reactive oxygen species (ROS) generation (chiefly superoxide anions, hydrogen peroxide, hydroxyl radicals) and natural cell antioxidant capacity in favor of the former**. (Frei,1994).** ROS can regulate many signalling pathways, such as infiltration of monocytes in intima and vascular smooth muscle cell proliferation. The cause of oxidative stress observed in atherosclerosis awaits clarification. Recent findings have suggested that the major source of ROS in the vascular wall, and also in vascular smooth muscle cells, is the NAD(P)H oxidase system. This is a membrane-associated enzyme, composed of five subunits, catalyzing the one-electron reduction of oxygen, using NADH or NADPH as the electron donor. NAD(P)H oxidase generates significant amounts of superoxide radicals, and an association between enzymatic activity and clinical risk factors in atherosclerosis has been shown**. (Guzik et al, 2000)**

Dietary advice has a small but significant role to play in normalizing abnormal serum lipids in those at high risk of cardiovascular disease and may lead to reduction in serum total cholesterol levels of the order of 3% to 6% are to be expected **(Clarke et al, 1997, Tang et al, 1998).** Replacement of saturated fats by unsaturated fats leads to improved lipid levels **(Mensink et al, 1992).** Fat is an important constituent of diet and regulates serum and hepatic lipid levels. Amount and type of dietary fat have a strong influence on serum TC level.

Safflower oil (SO) is belonging to the class of Semi-drying oils and it contains glycerides of palmitic (6.5%), stearic (3.0 %), arachidic (0.296 %), oleic (13.0 %), linoleic (76-79 %) and linolenic acids (90.15 %). The polyunsaturated fatty acid content of the SO is highest (75%) and is said to responsible to control cholesterol level in blood and thereby reduce incidence of heart attacks **(Kokate et al, 2005).**

Rice bran oil (RBO) is belonging to the class of non drying oil and it incorporates a rich unsaponifiable fraction mainly composed by phytosterols, 4 methyl- sterols tocotrienols, tocopherol and triterpene alcohols. It contains mainly oleic acid (38.4%),

linoleic acid (34.4%) and α-linolenic acid (2.2%) as unsaturated fatty acids, and palmitic (21.5%) and stearic (2.9%) acids as saturated fatty acids **(Sayre et al, 1990).**

It is reported that diets rich in certain saturated fatty acids (SFA) raise LDL-C compared with those observed on polyunsaturated fatty acids (PUFA) rich diets. **(Khosla et al, 1996)** SFA raises but PUFA lower TC and LDL-C. **(Mensink et al, 1992)** Phytosterols, tocopherols and tocotrienols are supposed to contribute to antihyperlipidemic action of rice bran and the derived oil, while the particular fatty acid mono- and polyunsaturated composition seems not to be fundamental in its activity. **(Rong et al.1992)**

Triton WR-1339 can produce dose related hyperlipidemia in a short period of time. The nonionic surfactant, triton WR-1339 has been used in lipid metabolism research to inhibit the removal of lipoprotein from the circulation. **(Borensztajn et al, 1976)** Triton WR-1339 is a nonionic detergent that prevents catabolism of triacylglycerol-rich lipoproteins by lipo-protein lipase and is commonly used for in vivo determination of triacylglycerol production and VLDL secretion or clearance rate. **(Schurr et al, 1972, Hayashi et al, 1981).** In the present study, intraperitoneal injection of Triton-WR-1339 at a dose of 300 mg/kg was used to induce hyperlipidemia in rats. The significant rise in the serum lipid profile such as elevated levels of TC, TG, LDL-C and VLDL-C were seen after 24 hrs triton-WR-1339 administration as compared to normal control group. Moreover, decline in HDL-C levels was seen in triton-WR-1339 control group. Triton WR-1339 also increased the oxidative stress in animals. These results demonstrate the feasibility of using triton WR-1339 to induce acute hyperlipidemia in rats. Atorvastatin (2 mg/kg, Orally) has also been included in the study in order to understand how far SO, RBO and various combinations of SO and RBO are comparable to that of a standard drug.

The results of the present investigation reveal that pretreatment of SO (7 ml/kg, Orally) to the hyperlipidemic rats caused a significant decrease in the serum TC and LDL cholesterol as compared to the triton WR-1339 control rats and it showed a non-significant decreased in the serum TG & VLDL level and

non-significant increase in the level of HDL compared to triton WR 1339 control group.

The reduction in the total cholesterol level by SO may be associated with the presence of high amount of linoleic acid which is polyunsaturated fatty acid. **(Suzuki et al, 1970, Oshima et al, 1970).** It is reported that Acyl-CoA cholesterol acyl transferase (ACAT) activity gets decrease by safflower seed ethanol extract (0.15%, wt/wt; SSE), which may lead to less cholesteryl ester being available for VLDL packing thereby resulting in a reduction of plasma cholesterol **(Carr et al,1992).** It is also reported that decrease in the HMG-CoA reductase mRNA expression, increase in the hepatic LDL receptor and/or cyp7 mRNA expression occurs by SO diet **(Watanabe et al, 2000).** The reduction in the LDL level by SO may be associated with the presence of PUFA which shows increase in the LDL apolipoprotein B (apoB) fractional catabolic rates (FCR). **(Shepherd et al, 1980)**

The results of the present investigation reveal that pretreatment with RBO (7 ml/kg, Orally) to the hyperlipidemic rats causes a significant decrease in the serum TC,TG,LDL and VLDL level as compared to the triton WR-1339 control rats. RBO also showed significant increase in the HDL cholesterol level as compared to the triton WR-1339 control rats.

The reduction in the total cholesterol level by RBO may be associated with the presence of phytosterols which act at the intestinal level by interfering with the absorption of the cholesterol from the gut **(Narasinga Rao BS, 2000).** The presence of cycloartenol, a triterpene alcohol, which shows a similar structure to cholesterol may compete with the binding sites of cholesterol and thus sequestering cholesterol from the system. The presence of tocotrienols which are thought to inhibit the HMG Co A reductase activity in the biosynthetic pathway of cholesterol**. (Raghuram et al,1989)**

The reduction in the triglyceride level by rice bran oil may be associated with the presence of triterpene alcohols and phytosterols which lowers the circulating levels of cholesterol and triglyceride possibly due to the structural similarity of cycloartenol and cholesterol **(Rukmini et al,1991).** The increase in the LPL (Lipoprotein lipase) which is capable of breaking down plasma triglycerides of TG-rich lipoproteins,

including chylomicrons and very low density lipoproteins (VLDL). **(Gotoda et al, 1989)**

The reduction in the LDL level and increase in HDL level by RBO rice bran oil may be associated with presence of ferulic acid (4- hydroxy-3–methoxy cinnamic acid) esters of triterpene alcohols and plant sterols. **(Rogers et al, 1993).** The increase in HDL level by RBO may be due to the increase in Lecithin cholesterol acyl transferase (LCAT) which plays a central role in cholesterol uptake by HDL particles from the peripheral tissues and maturation of HDL to cholesterol ester- rich HDL2 particles. (**Genest et al, 1999)**

Atherosclerotic index (A.I) is the major risk factor in the cardiovascular disease. It indicates the deposition of foam cells or plaque or fatty infilteration or lipids in heart, coronaries, aorta, liver and kidneys. The higher the atherosclerotic index, the higher is the risk of above organs for oxidative damage. **(Mehta et al, 2003).**

There was a significant increase in the atherosclerotic index in triton WR 1339 control groups. Treatment with SO showed significant decrease in the atherosclerotic index. There was a significant decrease in the HDL/TC ratio in triton WR 1339 control group. Treatment with SO showed a non-significant increase in the HDL/TC ratio. Treatment with RBO showed significant decrease in the level of the atherosclerotic index and showed a significant increase in the HDL/TC ratio.

The high content of tocopherols and tocotrienols in RBO may improve the oxidative stability of the blends of SO and RBO. In addition to improving the plasma lipid profile, blending of RBO with SO can result in an economic advantage of lower prices. **(Sunitha et al, 1997)** Administration of various combinations of SO and RBO in 2:8, 3:7, 5:5 proportion showed significant decrease in the serum TC,TG,LDL,VLDL, A.I level as compared to the triton WR-1339 control rats and showed significant increase in the HDL cholesterol , HDL/TC ratio as compared to the triton WR-1339 control.

Maximum depression in the TC level was seen in 5:5 proportion, which is significantly different from SO and RBO treated group, not significantly different from atorvastatin treated group. There was no significant change observed in the inter-combinations of SO and RBO. It may be associated with the presence of linoleic acid in SO and its effect on ACAT activity, HMG-CoA reductase mRNA expression, hepatic LDL receptor and/or cyp7 mRNA expression as well as presence of phytosterols, cycloartenol and tocotrienols in RBO.

Maximum depression in the TG & VLDL level was seen in 3:7 proportion, which is significantly different from SO treated group, not significantly different from RBO and atorvastatin treated group. There was no significant change was observed in the inter-combinations of SO and RBO. It may be associated with the presence of high amount of polyunsaturated fat which facilitated the interaction of the lipoprotein triacylglycerol with Lipoprotein lipase (LPL) resulting in a more rapid removal of the triacylglycerol from blood **(Engelbergh , 1966) ,** presence of triterpene alcohols and plant sterols in RBO, increase in the LPL activity. **(Gotoda et al, 1989)**

Maximum depression in the LDL level was seen in 2:8 proportion, which is significantly different from RBO treared group, not significantly different from SO and atorvastatin treated group. There was no significant change was observed in the inter-combinations of SO and RBO

Maximum increase in the HDL level was seen in 3:7 proportion, which is significantly different from SO, not significantly different from RBO and atorvastatin treated group. There was no significant change was observed in the inter-combinations of SO and RBO.

Maximum depression in the Atherosclerotic index level was seen in 2:8 proportion, which is not significantly different from SO, RBO and atorvastatin treated group. There was no significant change was observed in the inter-combinations of SO and RBO.

Maximum increase in the HDL/TC level was seen in 3:7 proportion, which is significantly different from SO but not significantly different from RBO and atorvastatin treated group. There was no significant change was observed in the inter-combinations of SO and RBO.

Increased intracellular generation of reactive oxygen species (ROS) plays an important role in chronic inflammatory responses to atherosclerosis. **(Chisolm et al, 2001).** ROS are generated in aerobic organisms during physiological or physiopathological oxidative metabolism of mitochondria. ROS may react with a variety of biomolecules, including lipids, carbohydrates, proteins, nucleic acids, and macromolecules of connective tissue, there by interfering with cell function. Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and antioxidant defense systems. Impairment in the oxidant/antioxidant equilibrium provokes a situation of oxidative stress and generally results from hyperproduction of ROS. Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases**. (Valko et al, 2007).**

A lot of oxygenated compounds, particularly aldehydes such as malondialdehyde (MDA) and conjugated dienes, are produced during the attack of free radicals to membrane lipoproteins and polyunsaturated fatty acids. Enzymic superoxide dismutase (SOD) and glutathione peroxidise (GSH-Px) and nonenzymic antioxidants play an important role in alleviating tissue damage due to the formation of free radicals. A lot of studies have found that serum MDA are higher in subjects with hyperlipidemia and decrease following dietary supplementation with antioxidants. **(Minhajuddin et al, 2005, Yang et al, 2006).**

MDA is an end product of peroxidative decomposition of polyenoic fatty acids in the lipid peroxidation process and its accumulation in tissue is indicative of the extent of lipid peroxidation. **(Draper et al, 1990)**

Glutathione, a potent inhibitor of the neoplastic process, plays an important role in the endogenous anti oxidant system. It is found in particularly high concentration in the liver and is known to have a key function in the protective process. Excessive production of free radicals resulted in oxidative stress, which leads to damage to

macromolecules. Reduced glutathione, a free radical scavenger protects cell and tissue structures including activation of T cells and macrophage and regulation of immune function **(Fidelus et al, 1986).** free radicals are the signalling entities in T cell activation. Reduced glutathione (GSH) act as anti-oxidants by reacting with free radicals and thus interrupting the propagation of new free radical species.

Treatment with SO, RBO and various combinations of SO and RBO showed significant reduction in the level of lipid peroxidation (MDA) as compared to triton WR 1339 control group, indicates the effective anti-oxidant property of the SO, RBO and various combinations of SO and RBO

Treatment with RBO and various combinations of SO and RBO showed significant increase in the GSH level as compared to triton WR 1339 control group indicates the effective anti-oxidant property of RBO and various combinations of SO and RBO whereas SO showed a non-significant increase in the GSH level.

The anti-oxidant activity of SO may be due to the direct radical and lipid peroxide scavenging actions. **(Harper et al., 1999, Ng et al., 2000)** and synergistic interaction with other antioxidants **(Nagata et al., 1999)**, preventing oxidative attack on membrane lipids by sparing vitamin E **(Gey et al, 1989),** inhibition of lipoxygenases. **(Agarwal et al., 1991, Capdevila et al., 1988)**.

The anti-oxidant activity of RBO may be due to the presence of oryzanol which has been shown to inhibit linoleic acid oxidation. **(Yagi et al , 1979, Xu et al ,2001)** and cholesterol oxidation.(**Xu et al , 2001).** The presence of oryzanol components, namely, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate also act as antioxidants in methyl linoleate bulk and multiphase lipid systems and as radical scavengers. **(Kikuzaki et al , 2002).**

Maximum depression in the MDA level was seen in 2:8 proportion but it is not significantly different from SO, RBO and atorvastatin treated group. There was no significant change was observed in the inter-combinations of SO and RBO.

Maximum increase in the GSH level was seen in 5:5 proportion, which is significantly different from SO but not significantly different from RBO and atorvastatin treated group. There was no significant change was observed in the inter-combinations of SO and RBO.

It appears that antioxidant activity of the combinations of safflower oil and rice bran oil may be partly associated with the direct radical and lipid peroxide scavenging actions, synergistic interaction with other antioxidants, preventing oxidative attack on membrane lipids by sparing vitamin E and inhibition of lipoxygenases.

7. CONCLUSION:

In the present study, maximum depression in Total cholesterol was seen in [SO + RBO] (5:5) proportion, maximum depression in Triglyceride and VLDL and maximum increase in HDL and HDL/TC was seen in [SO + RBO] (3:7) proportion, maximum depression in LDL and Atherosclerotic (A.I) was seen in [SO + RBO] (2:8) proportion. Maximum depression in Malondialdehyde (MDA) was seen in [SO + RBO] (2:8) proportion and maximum increase in Reduced glutathione (GSH) was seen in [SO + RBO] (5:5) proportion.

So, our study suggests that SO and RBO in 3:7 proportion is the optimum combination as it shows the maximum depression in TG and VLDL level and it shows the maximum increase in HDL and HDL/TC which may be beneficial in treatment of hyperlipidemia. However further study is required to confirm its mechanisms for the higher effect as compared to other combinations.

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9. APPENDIX:-

LIST OF INSTRUMENTS:-

INSTRUMENTS	SOURCE
UV Spectrophotometer	ELICO SL- 164
Homogeniser	Remi Motors Pvt. Ltd
Centrifuge	Remi Motors Pvt. Ltd.
Electronic Balance	Lab Series C-3, Roy Electronics
pH Meter	LI 127, Elico Ltd.
Micropipette	Accupette
Hot Air Oven	Elico Ltd.
Microtips	Tarsons Ltd.
Aluminium foils	Nice Chemicals Ltd.

LIST OF CHEMICALS:-

CHEMICAL	SOURCE
Triton WR 1339	Himedia chemicals, Mumbai
Rat Diet	PranavAgro, Pune
Diagnostic kits	Lab Care Dianostics Pvt. Ltd.
Dithiobisnitro benzoic acid	Sisco Research Lab.

All other chemicals were purchased from CDH Ltd., New Delhi.